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Molecules and Cells

Engineering of Flavonoid *O*-Methyltransferase for a Novel Regioselectivity

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An *O*-methyltransferase isolated from poplar, POMT7, was identified as a flavone 7-*O*-methyltransferase. In order to generate a mutant of POMT-7 having a novel regioselectivity, we conducted an error-prone polymerase chain reaction. More than 100 mutants were screened and one of the mutants (POMT-M1) Asp257Gly, methylated the 3-hydroxyl group of flavonols in addition to 7-hydrdoxyl group. The mutation changed asparagine residue at the position of 257 into glycine. The kinetic parameters showed that the wild type POMT7 was better activity toward kaempferol and quercetin than the POMT7-M1. Using *E. coli* transformant expressing *POMT7-M1*, 58 μM of 3, 7-*O*-dimethylquercetin and 70 μM of 3, 7-*O*-dimethylkaempferol from 100 μM of corresponding substrate were synthesized successfully.

INTRODUCTION

Secondary metabolites from plants- most notably the alkaloids, phenylpropanoids, and terpenoids- exert a variety of biological functions on plants as well as human (Croteau et al., 2000). Starting from several basic structures, these secondary metabolites undergo various modification reactions, resulting in overwhelming number of metabolites. For example, more than 12,000 alkaloids and 10,000 flavonoids have been isolated (Croteau et al., 2000; Tahara, 2007).

Flavonoids originated from the phenylpropanoid pathway form a variety of subgroups; flavanone, flavone, flavonol, isoflavone, anthocyanidine, and flavane (Passamonti et al., 2009). Several oxygenases, including cytochrome P450 and dioxygenases mediate the biosynthesis of these compounds (Winkel-Shirley, 2001). In addition, modification reactions, such as *O*-methylation and *O*-glycosylation have been shown to be prevalent (Ibrahim et al., 1998; Jones and Vogt, 2001).

The *O*-methylation of flavonoids is a common modification reaction observed in flavonoid biosynthesis in plants. The analysis of flavonoids from plants has revealed a variety of *O*-methylated flavonoids. The *O*-methyltransferases (OMTs) are in charge of transferring a methyl group from *S*-adenosylmethione to the hydroxyl group of various substrates including flavonoids. Several studies have been conducted on flavonoid *O*-methyltransferases that evidenced differing regioselectivity and reactivity (reviewed by Lam et al., 2007). In addition, the structures of flavonoid OMTs from several organisms have been elucidated (reviewed by Ferrer et al., 2008). OMTs in flavonoid biosynthesis can be categorized into two classes; low molecular weight (23-27 kDa), metal dependent OMTs (Class I) and high molecular weight (40-43 kDa), metal independent OMTs (Class II) (Kopycki et al., 2008). Although class I OMTs mediate a methyltransfer reaction in monolignol modification reactions, the subgroup of class I OMTs utilizes not only monolignols but also flavonoids. Typical flavonoid OMTs belong to class II (Kopycki et al., 2008). The expression of OMT genes in heterologous system such as *Escherichia coli*, based on the sequences retrieved by various genome project as well as biochemical characterization using classical enzyme purification methods, contributed to the elucidation of the reactivity and regioselectivity of OMTs. With quercetin as a standard flavonoid for the *O*-methylation reaction, thus far, 7, 3′, and 4′ specific OMTs have been cloned from different plants by our groups in addition to others (Deavours et al., 2006; Kim et al., 2005; 2006a; 2006b; Muzac et al., 2000). Flavonoid 3-*O*-methyltransferase was purified from *Serratula tinctoria* (Huang et al., 2004) but the corresponding gene has not been cloned. Thus far, there have been no attempts to change the regioselectivity of flavonoid *O*-methyltransferases.

POMT7 was cloned from poplar and encoded a flavone 7-*O*-methyltrasnferase from poplar (Kim et al., 2006b). Its regioselectivity was altered from 7-hydroxyl to the 4′-hydroxyl group when it utilized 3′-*O*-methylflavones such as 3′-*O*-methylluteolin (Kim et al., 2008a). In order to generate an OMT with a novel regioselectivity from POMT-7, we carried out error-prone polymerase chain reaction (PCR). After screening one hundred-fifty clones, an OMT with a new regioselectivity was isolated. The mutant evidenced a 3-*O*-methyltransferase activity as well as 7-*O*-methyltransferase activity of flavones. With *E. coli* harboring this mutant *POMT-7*, we were able to synthesize the 3,7-*O*-dimethylquercetin and 3,7-*O*-dimethylkaempferol.

MATERIALS AND METHODS

Error-prone polymerase chain reaction

POMT7 cloned into pGEX vector was used as a template. The pGEX5′ and pGEX3′ primers (Amersham, USA) were used as primers. Two different deoxynucleotides mixtures were used; mixture I consisted of 250 μM of dATP and dTTP and 50 μM of

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dCTP and dGTP while mixture II consisted of 250 μM of dCTP and dGTP and 50 μM of dATP and dTTP. PCR was carried out 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C using taq DNA polymerase (Bioneer, Korea). The resulting PCR product was purified using a PCR product purification kit (Bioneer) and digested with BamHI/NotI. The resulting DNA was ligated with the corresponding sites of pGEX vector and transformed into *E. coli* BL21.

Screening of a novel *O*-methyltransferase and biotransformation of kaempferol and quercetin expressing *POMT7* mutant

Each clone from the error prone PCR was grown in 3 ml of LB medium containing 50 μg/ml ampicilin and induced via the addition of IPTG at the final concentration of 0.1 mM. Quercetin was then added to the medium at the concentration of 100 μM. Each mixture was incubated for 15 h at 25°C with shaking. An equal volume of ethylacetate was added to each mixture. The ethylacetate was evaporated and dissolved in methanol, which was then analyzed with thin layer chromatography (TLC; Yoon et al., 2005) or high performance liquid chromatography (HPLC; Kim et al., 2009).

E. coli transformant harboring POMT7 Asp257Gly mutant (POMT7-M1, see below) was used for the production of 3,7-*O*-dimethylquercetin and 3,7-*O*-dimethylkaempferol. Transformant was induced as described above. The cells were harvested by centrifugation and resuspended in LB medium containing 50 μg/ml ampicillin. The substrates, kaempferol or quercetin were added at a final concentration of 100 μM. The mixture was incubated at 25°C with careful shaking for 24 h, and periodically harvested. The resulting samples were extracted twice with equal volume of ethylacetate and dried by speed vac. The dried sample was dissolved in 70 μl of DMSO and analyzed using HPLC (Kim et al., 2009).

Nuclear magnetic resonance spectroscopy of the methylation compounds was conducted as described in Kim et al. (2006a). The ^{1}H NMR data of reaction products of quercetin and kaempferol were assigned. Its ^{1}H NMR data are as follows: Quercetin reaction product: ^{1}H-NMR (400 MHz, DMSO-d6): δ 3.79 (3H, s, H-3-OCH_3), 6.37 (^{1}H, d, J = 2.0 Hz H-6), 3.86 (^{3}H, s, H-7-OCH_3), 6.69 (^{1}H, d, J = 2.1 Hz, H-8), 7.58 (^{1}H, d, J = 2.2 Hz, H-2′), 6.94 (^{1}H, d, J = 8.5 Hz, H-5′), 7.48 (^{1}H, dd, J = 8.4, 2.2 Hz, H-6′). Kaempferol reaction product: ^{1}H-NMR (400 MHz, DMSO-d6): δ 3.80 (^{3}H, s, H-3-OCH_3), 12.68 (^{1}H, s, H-5), 6.38 (^{1}H, d, J = 2.1 Hz, H-6), 3.86 (^{3}H, s, H-7-OCH_3), 6.75 (^{1}H, d, J = 2.2, H-8), 7.98 (^{2}H, d, J= 8.8, H-2′/H-6′), 6.94 (^{2}H, d, J = 6.95, H-3′/H-5′). ^{1}H NMR data of each reaction product matched with previously reported ^{1}H NMR data (Guerreo et al., 2002; Stevens et al., 1995). Mass spectrometry (MS) experiments were carried out as describe in Lim et al (2008).

Molecular modeling of POMT7

Templates for modeling were obtained from a BLAST search and crystallographic structures were obtained from the Protein Data Bank. The homology modeling software PRIME incorporated into the Schrodinger modeling software suite was used to generate a structure of the POMT7 homologous to the crystallographic structure of isoflavone *O*-methyltransferase (PDB ID: 1FP2). The crystallographic positions of the backbone atoms and conserved side chains were mapped from the template. The side chain coordinates for all non-identical residues were predicted using PRIME. The optimal model was selected based on bond angle stereochemistry using PROCHECK. After refinement of the loop structures, the chosen model was subjected to energy minimization and molecular dynamics simulations (MD) in order to obtain a stable, low-energy conformation. Energy minimization was performed using a conjugate gradient minimize (0.05 convergence criteria), the OPLS-AA force field, and GB/SA continuum water model. MD simulations were performed by pre-equilibration for 100 ps and simulation for 1 ns at 300 K with a 1-fs time step and SHAKE applied to all bonds to hydrogen. The root mean square deviation (RMSD) between the main chain atoms of the models and the template was calculated for the reliability of the models. Finally, the best quality models of POMT7 were chosen for docking the ligands in order to determine the binding mode of flavonoids. We used the protein preparation utilities in Maestro to assign the charge state of ionizable residues, add hydrogens, and carry out energy minimization. The ligand quercetin was then docked into the homology models using GLIDE (http://www.schrodinger. com). The default setting of the extreme precision mode of GLIDE was employed for the docking, and up to ten poses were saved for analysis. All of the saved poses were similar and therefore, the top scored pose was chosen for the binding mode analysis.

Recombinant protein purification and enzyme assay

The recombinant POMT7 and mutants were purified using GST affinity column chromatography (GST trap, Amersham Bioscience, USA). The purification procedure followed the manufacturer's instructions.

To measure the kinetic parameters of wild type POMT7 and POMT7-M1, a reaction mixture was prepared containing 20-34 μg of the purified recombinant protein, 300 μM *S*-adenosylmethionine (SAM) and 10-200 μM substrate range in 10 mM Tris/HCl buffer (pH 7.5) at a final volume of 200 μl. The reaction mixture was incubated at 37°C for 1 h, stopped with 10 μl of 5 N HCl, extracted twice with equal volume ethylacetate and the organics layer was evaporated using vacuum dryer. All reaction was repeated three times. Reaction products were also analyzed by HPLC (Varian, USA) equipped with a photodiode array detector using a C18 reversed-phase column (Varian, 4.60 mm × 250 mm, 3.5 um particle size). HPLC analysis program followed as described in Kim et al. (2009).

RESULTS AND DISCUSSION

Creation of a new POMT7 mutant by error prone polymerase chain reaction

Error-prone PCR has been applied in efforts to improve the reactivity or alter the specificity of the enzyme (Jaeger et al., 2001). This approach was applied in order to alter the regioselectivity of POMT7. One-hundred fifty clones were screened. Initial TLC analysis demonstrated that approximately 100 clones yield reaction product, whereas approximately 50 clones did not give any product. The TLC method we used in this study was capable of separating quercetin from *O*-methylated quercetin, but the position of methylation could not be distinguished. Thus, those clones producing a reaction product were analyzed further using HPLC. Among them, one clone, GC1 produced three peaks. One of them (P2 in Fig. 1B) had the same retention time to 7-*O*-methylquercetin. P3 and P4 had different retention times from those of either quercetin itself or 7-*O*-methylquercetin. The MS analysis of two products showed that the molecular mass of P3 was increased by 14-Da and that of P4 was increased by 28-Da, indicating that P3 was monomethylated and P4 was dimethylated.

Both strands of the mutant GC1 were sequenced in order to determine the location of mutation(s). Two nucleotides were mutated in GC1 as compared to the wild type; T to A at 301 and A to G at 770. The first mutation resulted in change of Leu

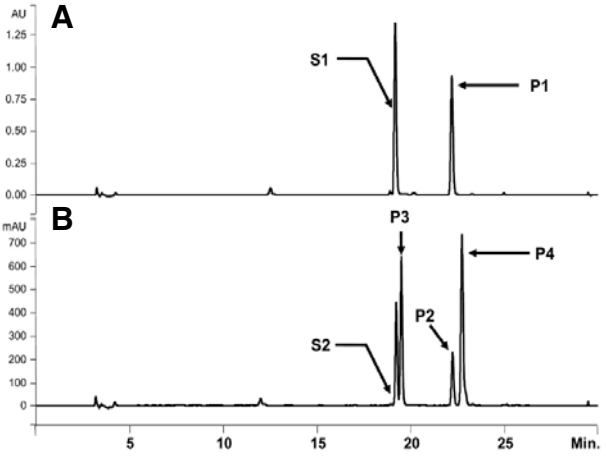


Fig. 1. HPLC analysis of quercetin with mutant GC1. (A) Reaction product of quercetin with wild type POMT7. S1, quercetin; P1, 7-*O*-methylquercetin. (B) Reaction product of quercetin with GC1. S2, quercetin; P2, 7-*O*-methylquercetin; P3, 3-*O*-methylquercetin; P4, 3,7-*O*-dimethylquercetin.

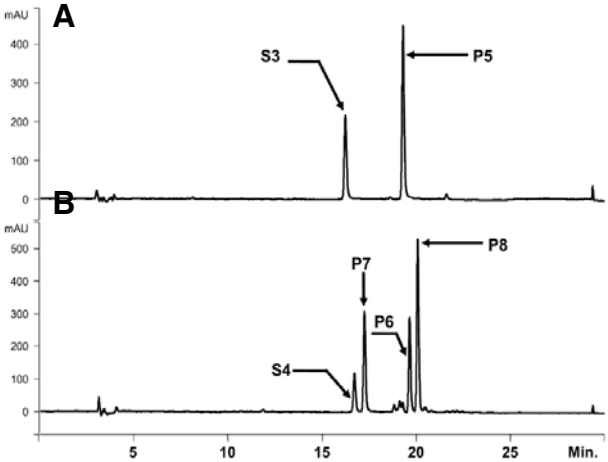


Fig. 2. HPLC analysis of kaempferol with POMT7 wild type and mutant POMT-M1. (A) Reaction product of kaempferol with wild type POMT7. S3, kaempferol; P5, 7-*O*-methylkaempferol. (B) Reaction product of kaempferol with GC1. S4, kaempferol; P6, 7-*O*-methylkaempferol; P7, 3-*O*-methylkaempferol; P8, 3,7-*O*-dimethylkaempferol .

to Met at 101 and the second caused a change from Asp to Gly at 257. Two mutations were separated to evaluate the role of each mutation in the production of new methylated quercetin. Because a unique restriction enzyme site, Xba I is located between two mutations, each mutation was easily separated. Each of them was then subcloned into pGEX 5X-1 *E. coli* expression vector and expressed in *E. coli*. Two mutants, as well as the wild type were expressed as soluble protein in *E. coli*. The reaction of quercetin with the Leu101Met mutant revealed the same reaction product to the wild type POMT7 (data not shown). On the other hand, Asp257Gly produced reaction product profiles indistinguishable from GC1, the double mutant. This suggested that Asp257Gly (POMT7-M1) contributed to the production of the new methylated quercetin.

The position of methylation in quercetin was determined by comparing the reaction product with an authentic compound or NMR. The P3 (Fig. 1B) had the same retention time with authentic 3-*O*-methylquercetin, suggested that P3 is a 3-*O*-methylquercetin. And, the structure of P4 was determined to be 3,7-*O*-dimethylquercetin by NMR (See "Materials and Methods").

Characterization of POMT7-M1

The POMT7-M1 was further characterized with other flavonoid containing 3 and 7 hydroxyl group. Kaempferol was used as a substrate. Analysis of kaempferol reaction product incubated with POMT7-M1 using HPLC evidenced two new peaks (P7 and P8 in Fig 2B.), which was not noted when wild type POMT7 reacted with kaempferol. The molecular mass of the two new reaction products was increased by 14 and 28-Da, respectively, indicating that one methyl group was attached to one product (P7) and two methyl groups were to the other product (P8), just like quercetin. In a single methylated reaction product from kaempferol or quercetin, the methylation position was 3-hydroxyl groups based on the comparison of retention time with authentic 3-*O*-methylkaempferol. The molecular structures of dimethylated products from kaempferol were determined using NMR. The proton NMR comparison of the reaction products with authentic compounds identified dimethylated product as 3,7-*O*-dimethylkaempferol ("Material and Methods").

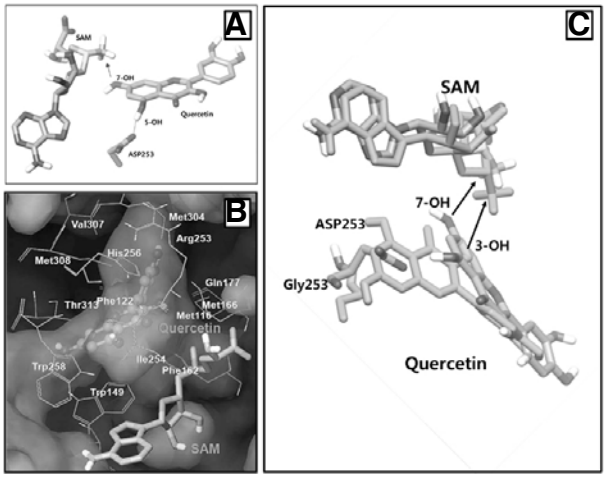
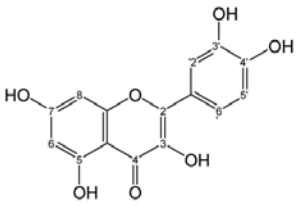


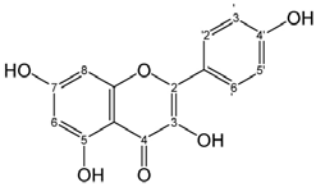
Fig. 3. (A) Substrate binding pocket of wild type POMT7. Molecular docking between quercetin and POMT7 revealed a specific interaction between quercetin and Asp257. (B) Substrate binding pocket of mutant POMT7-M1. Mutation of Asp257Gly resulted in the formation of a hydrophobic pocket into which quercetin fits. (C) The overlapping of substrate binging pockets from POMT7 (atom type) and POMT7-M1 (gray). Quercetin is positioned for 7-*O*-methylation in POMT7 while it is positioned for 3-*O*-methylation in POMT7-M1.

In order to examine the molecular basis of the new regioselectivity of Asp257Gly mutant, molecular docking of quercetin into the modeled structure of Asp257Gly was conducted. In the wild type POMT-7, the A ring of quercetine was positioned close to the SAM. Additionally, Asp257 formed a hydrogen bond with 5-hydroxyl group of quercetin (Fig. 3A). This resulted in an *O*-methylation at the 7-hydroxyl group of quercetin. In the mutant Asp257Gly, the overall structure of quercetin binding site was altered; the C ring of quercetin is located close to the SAM. Quercetin fits into the hydrophobic pocket formed by Met304, Val307, Arg253, His256, Met308, Trp258, Thr313, Phe122, Ile254, Gln177, Trp149, Phe162, Met116, and Met166

Table 1. Substrate specificity of wild type POMT7 and POMT7-M1



Quercetin



Kaempferol

Enzyme	Substrate	K_m (μM)	Vmax (nk_{cat}/mg)	Vmax/K_m	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
POMT7	Quercetin	29.8 ± 1.6	110 ± 17.9	3.7 ± 0.4	0.071 ± 0.016	$2.4 \times 10^{-3} \pm 0.28$
	Kaempferol	32.4 ± 2.5	79 ± 15.3	2.4 ± 0.3	0.064 ± 0.02	$1.9 \times 10^{-3} \pm 0.57$
POMT7-M1	Quercetin	32.4 ± 1.6	73.1 ± 5.0	2.3 ± 0.4	0.064 ± 0.018	$1.9 \times 10^{-3} \pm 0.54$
	Kaempferol	37.8 ± 4.8	61.6 ± 7.9	1.5 ±0.2	0.07 ± 0.009	$1.7 \times 10^{-3} \pm 0.2$

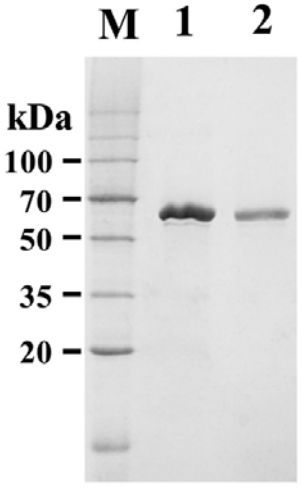


Fig. 4. Purification of the recombinant POMT7 and POMT7-M1. About 1.0-1.5 μg protein purified by affinity column chromatography was loaded on gel. M, standard protein size marker; 1, POMT7; 2, POMT7-M1.

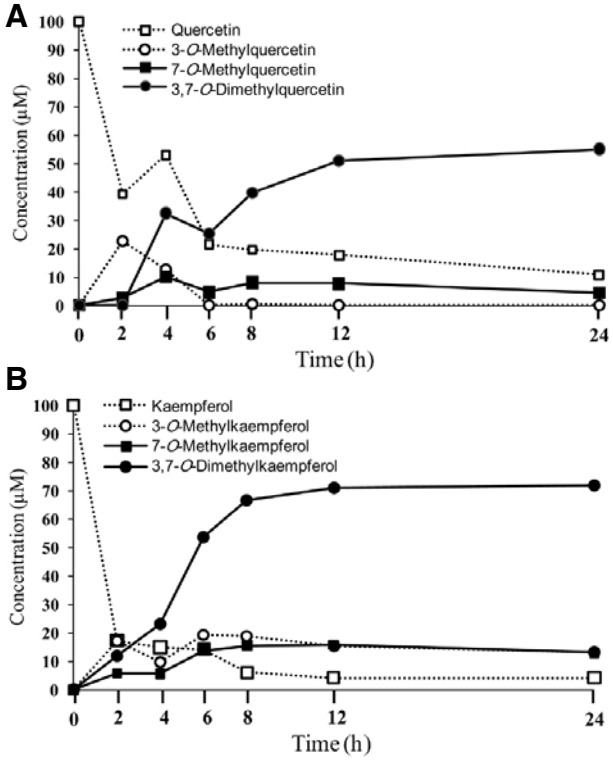


Fig. 5. Biotransformation of quercetin (A) and kaempferol (B) using *E. coli* harboring *POMT7-M1*.

(Fig. 3B). Thus, the most favorable configuration between POMT7-M1 and quercetin was the one in which the 3-hydroxyl group of quercetin was positioned close to the methyl group donor, SAM. The overlapping of substrate binging pockets from POMT7 and POMT7-M1 clearly showed different positioning of quercetin toward SAM (Fig. 3C). These results indicated that only one amino acid change in POMT7 could result in a novel regioselectivity. It has been several reports that OMTs having high amino acid sequence identities have different reactivity. OMT II-1 and OMT II-4 from *Thalictrum tuberosum* showed a single amino acid difference among 365 amino acid but they showed different reactivity for several substrates (Frick and Kutchan, 1999). OMT1 and OMT2 from *Chrysosplenium americanum* (Gauthier et al., 1998) also showed high amino sequence identity (only three amino acid difference among 381 amino acids). However, OMT1 showed higher activity for luteolin and quercetin than OMT2. These results showed that a few amino acid differences could result in the difference in activity. However, thus far, there has been no report showing that changes in a few amino acids of OMTs resulted in the alternation of regioselectivity. Our study is the first report about alternation of regioselectivity by a change of one amino acid.

Both *POMT7* and *POMT7-M1* were expressed as GST-fusion protein in *E. coli* and the recombinant proteins were purified (Fig. 4). Kinetic parameters of wide type POMT7 and POMT7-M1 for kaempferol, and quercetin were determined using Lineweaver-Burk plots (Table 1). The relative activity of two enzymes was compared using k_{cat}/K_m. The wild type POMT7 showed approximately 26% the better affinity toward kaemp-ferol and 12 % toward quercetin than POMT7-M1.

Biotransformation of kaempferol and quercetin using *E. coli* expressing *POMT7-M1*

The conversion of kaempferol or quercetin into the correspond-

ing 3,7-*O*-dimethylates was carried out using *E. coli* harboring *POMT7-M1*. These two methylated flavonoids were shown to contain several biological activities including anti-inflammatory, antinociceptive, and vasorelaxant activities (Guerrero et al., 2002; Kim et al., 2008b; Küpeli and Yesilada, 2007).The concentration of quercetin dropped continuously. 3-*O*-methylquercetin appeared more rapidly than 7-*O*-methylquercetin. However, they are likely to be converted into 3,7-*O*-dimethylquercetin. After 24 h incubation, approximately 58 μM of 3,7-*O*-dimethylquercetin was produced. The conversion of kaempferol into 3,7-*O*-dimethylkaempferol was similar to that of quercetin. However, approximately 70 μM of 3,7-*O*-dimethylkaempferol was produced at the end of incubation (Fig. 5).

The sequential *O*-methylation of flavonoids by a single OMT has been previously noted, although the sequential *O*-methylation of flavonoids is something of a rare event. CrOMT2 from *Catharanthys roseus* methylated myricetin into 3′,5′-*O*-dimethylmyricetin with 3′-*O*-dimethylmyricetin as a possible intermediate (Cacace et al., 2003). In addition, TaOMT2 from *Triticum aestivum* methylated tricetin into 3′-*O*-methyltricetin, 3′, 5′-*O*-dimethyltricetin and eventually 3′,4′,5′-*O*-trimethyltricetin (Zhou et al., 2006). These OMTs are flavonid B-ring specific. The mutant we generated with POMT7 gained a new regioselectivity in a different ring. Thus far, no flavonoid OMTs evidenced a *O*-methylaton reactions in different ring. Thus, it is, to the best of our knowledge, the first report concerning the change the regioselectivity of OMT using error-prone PCR.

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