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Regiospecific Flavonoid 7-*O*-Methylation with *Streptomyces avermitilis O*-Methyltransferase Expressed in *Escherichia coli*

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O-Methylation, commonly found in synthesis of secondary metabolites of plants and micro-organisms, appears to transfer a methyl group to the hydroxyl group of the recipient which increases the hydrophobicity of the recipient. *O*-Methyltransferase (OMT), *SaOMT-2*, was isolated and characterized from *Streptomyces avermitilis* MA-4680. Its amino acid sequence showed 68% similarity with antibiotic C-1027 OMT and 53% similarity with the carminomycin 4-OMT. *SaOMT-2* was expressed in *E. coli* as a His-tag fusion protein and showed that the methyl was transferred onto the 7- hydroxyl group of the isoflavones, daidzein and genistein, and the flavones, kaempferol and quercetin, as well as the flavanone naringenin. NMR and liquid chromatography−mass spectrometry were used to confirm the location of the methyl group on the recipient compound of naringenin, which was biotransformed into sakuranetin by *E. coli* transformant expressing *SaOMT-2* (*E. coli* Sa-2). Therefore, *E. coli* Sa-2 would be used for the synthesis of the antifungal flavonoid, sakuranetin, through biotransformation.

KEYWORDS: Biotransformation; flavonoids; *O*-methyltransferase; *Streptomyces avermitilis*

INTRODUCTION

Flavonoids are among the most ubiquitous phenolic compounds found in nature. Biosynthesis of flavonoids is well characterized, and most of the genes involved have been characterized from several plants (*1*). Because of the significance of flavonoids on human health, the catabolism of flavonoids by intestinal micro-organisms or phenol group oxidants were the focus in other organisms (*2*). Other than the degradation of flavonoids, hydroxylation and methylation were shown to modify the flavonoid structure in several microorganisms including *Streptomyces* sp. (*3*−*6*), but none of the microbial enzymes or genes that are involved in flavonoid hydroxylation and methylation have been characterized.

In humans, flavonoids serve as antioxidants that protect against cardiovascular diseases and certain forms of cancer (*7*). Their roles in plants are diverse, including UV protection, activation of *Rhizobium* nodulation, and function as antimicrobial agents against pathogens (*1*). These functional diversities result mainly from the structural diversities that originate from various modification reactions. Typical flavonoid modification reactions are mediated by glycosyltransferases (GT), cytochrome P450, and *O*-methyltransferases (OMTs). These genes have been cloned and characterized from plants (*1*, *8*−*10*). With the advent of microbial genome projects, microbial genes from a diverse genre have been annotated and classified as GTs, P450s, or OMTs. *Streptomyces avermitilis* MA-4680 is one of the microorganisms whose genome project was completed (*11*−*12*). Several of its genes were annotated as P450s, GTs, and OMTs; however, we are interested in the modification of flavonoids with OMTs. OMT catalyzes the transfer of the methyl group of *S*-adenosyl-L-methionine to the hydroxyl groups of the acceptor compounds. *O*-Methylation is thought to play an important role in inactivating the reactive hydroxyl groups of flavonoids and altering their solubility and intracellular compartmentation. OMTs from plants showed specificity and the regiospecificity toward flavonoids and alkaloids (*13*). However, OMTs from microbes have not been well studied even though most micro-organisms contain OMT genes. So far, OMT from *Streptomyces coelicolor* was known to modify flavonoids with broad specificity (*14*). OMT genes from micro-organisms could have potential for biotechnological modification of several chemicals including flavonoids, alkaloids and antibiotics. In this study, we report the characterization and expression of 7-*O*-methyltransferase from *S. avermilitis* (*SaOMT-2*) which converts the isoflavones, daidzein and genistein, and the flavones, kaempferol, apigenin, and quercetin, as well as the flavanone naringenin, into the corresponding 7-*O*-methylated metabolites. Also, the *E. coli*-containing *SaOMT-2* was used to produce an antifungal flavonoid, sakuranetin, which showed that transgenic microorganisms could use to produce a bioactive compound.

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MATERIAL AND METHODS

Cloning and Expression of *SaOMT-2*.

S. avermitilis MA-4680 (ATCC 31267) genomic DNA was isolated with a Qiagen DNeasy tissue kit (Quiagen, Gaitherburg, MD). *SaOMT-2* was amplified by polymerase chain reaction (PCR), using the genomic DNA of *S. avermitilis* as the template under the following conditions: 40 cycles of 1-min denaturation at 94 °C, 1-min annealing at 50 °C, and 1-min amplification at 72 °C. The primers, which were designed based on the putative OMT of *S. avermilitis*, were 5′-ATCATATG-GCCAAGGAGACGACCCC-3′ as the forward primer and 5′-ATG-GATCCTCAGCCAACCGCGCGCAGTT-3′ as the reverse primer. Restriction sites *Nde*I at the forward primer and *Bam*HI site at the reverse primer (underlined), were attached to facilitate the cloning. The PCR product was purified and subcloned into pET15b (Novagen, USA) as *Nde*I/*Bam*HI fragments.

The OMT sequences were aligned using CLUSTALW (http://www.ebi.ac.uk/ clustalw). The BoxShade program (http://www.isrec.isb-sib.ch:8080/software/ BOX_form.html) was used to highlight conserved and similar amino acids.

ANALYSIS OF METABOLITES

Flavonoids and esculetin used in this study were purchased from Indofinechemicals (New Jersey, USA).

The *E. coli* Sa-2 grew until absorbance at 600 nm reached 0.8. At this point, IPTG was added at a final concentration of 1 mM, and the transformant was grown for 4 more hours at 25 °C. After induction, the cells were harvested and resuspended in the LB medium containing 50 μg/mL of ampicillin. Substrates were added in the final concentrations of 60 μM. The mixture was incubated on a shaker at 30 °C for 12 h. Analysis of the substrates and reaction products by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and liquid chromatography−mass spectrometry (LC-MS) were done as described in Kim et al (*13*). Quantification of metabolites and parent material over time was monitored using HPLC in duplicate experiments. Several different concentrations of each substrate were analyzed with HPLC, and the HPLC value was used as standard for the analysis of the remaining reaction product after biotransformation of substrates.

NMR SPECTROMETRY

The 500 mL of apigenin, naringenin, and quercetin reaction products with SaOMT-2 were extracted twice with an equal amount of ethyl acetate, respectively, and the organic layer was evaporated. Each reaction product was redissolved in 0.5 mL of DMSO-d_6 (Aldrich, Chicago, IL). The NMR spectra were obtained in DMSO-d_6 on a Bruker Avance 400 instrument (400 MHz, 9.4 T) (Karlsruhe, Germany). For the ^{1}H NMR experiment, 32 transients were acquired with the spectral width of 8 000 Hz. ^{13}C NMR experiments were carried out using 64K data points, with the spectral width of 22 700 Hz. The data were processed using Xwinnmr software provided by Bruker (Karlsruhe, Germany).

RESULTS AND DISCUSSION

Isolation and Expression of *SaOMT-2*.

Protein coding genes of the *S. avermitilis* MA-4680 (http://www.ncbi.nlm.nih.gov/genomes/altik.cgi?gi=294&db=Genome) was examined to find *O*-methyltransferases, and six putative OMTs were found. Two OMTs found in *S. avermitilis* consisted of approximately 280 amino acids, and the four OMTs consisted of approximately 360 amino acids. Primers covering six genes were designed, and full-length genes were amplified. These six genes were named *SaOMT-1* to *SaOMT-6* (Genbank accession number 1211872, 1213742, 1214056, 1215943, 1210103, 1216331). All of the SaOMTs were expressed in *E. coli* as a his-tag fusion protein, but the yield and purity of the expressed SaOMT-2 after purification was highest among the expressed SaOMTs. Thus, *SaOMT-2* was further studied. *SaOMT-2* consisted of a 1080-bp open-reading frame encoding a 37.5-kDA protein. Basic local alignment search tool (BLAST) analysis of SaOMT-2 showed 68% similarity with OMT from *S. globisporus*, which is involved in antibiotic C-1027 biosynthesis (*15*), 56% similarity with *S. purpurascens* in rhodomycin, and 53% similarity with *S. peucetius* in carminomycin (*16*), all of which have a cyclic polyketide backbone (**Figure 1**). In addition, it showed a homology with plant OMTs that methylate flavonoids (*17*, *18*). The recombinant SaOMT-2 was purified and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure 2**). The molecular weight of the purified recombinant SaOMT-2 was about 38 kDA, which was in agreement with the predicted molecular size of the SaOMT-2 plus extra amino acids for purification.

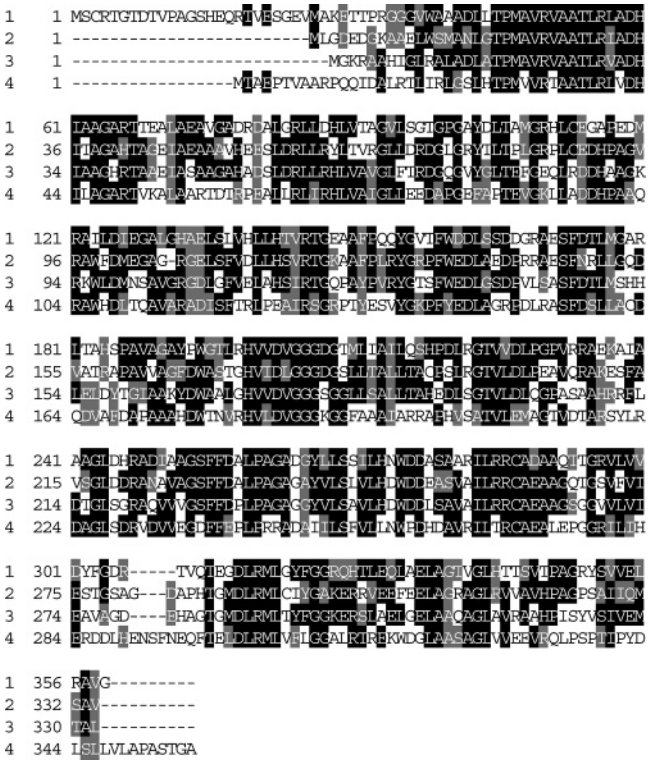


Figure 1. Amino acid alignment of *SaOMT-2* with other *O*-methyltransferases: 1, *O*-methyltransferase from *Streptomyces avermilitis* (SaOMT2); 2, *O*-methyltransferase from *Streptomyces globisporus* (gi:24575112); 3, *O*-methyltransferase from *Streptomyces carzinostaticus subsp. neocarzinostaticus* (gi:28192471); 4, carminomycin 4-*O*-methyltransferase from *Streptomyces sp.* (gi:530214).

Methylation of Flavonoids with SaOMT-2.

SaOMT-2 is predicted to modify a polyketide based on the sequence homology with other OMTs. Because polykedites share a common structural feature with flavonoids, several phenolic compounds, such as apigenin, caffeic acid, catechol, daidzein, esculetin, genistein, naringenin, kaempferol, and quercetin, were tested as potential substrates of SaOMT-2. In addition, recently a prenyltransferase from *Streptomyces.* sp strain CL190 had activity not only for polyketides but also for flavonoids, probably due to structural similarity (*19*). Instead of using the purified recombinant protein, biotransformation with

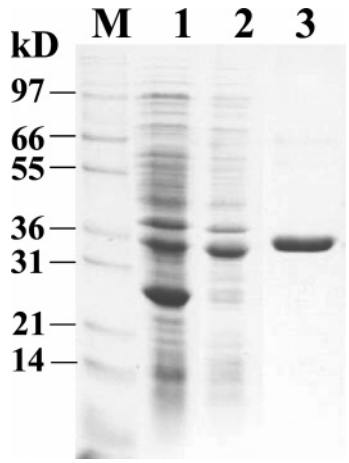


Figure 2. SDS-PAGE of the expressed recombinant SaOMT-2. M, Standard protein markers; 1, *E. coli* lysate before induction; 2, *E. coli* lysate after induction; 3, His tagged affinity purified protein

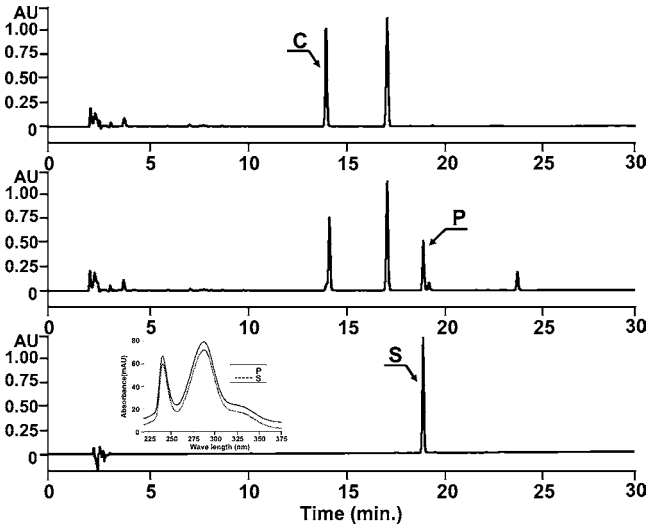


Figure 3. HPLC elution profile of the SaOMT-2 reaction products with naringenin. (C, naringenin; P, naringenin reaction product with SaOMT-2; S, authentic sakuranetin.) The peak at around 17 min is *E. coli* metabolites. Inset: UV spectra of SaOMT-2 reaction product (P) and authentic sakuranetin (S).

E. coli Sa-2 was carried out because it is more suitable for biotechnological application. Also, flavonoids that were used as substrates for *E. coli* Sa-2 could penetrate into *E. coli* cells so that they served as substrates for biotransformation (*20*, *21*).

The *E. coli* transformant containing *SaOMT-2* was incubated with 60 μM of each substrate. As a negative control, same experiment was carried out with *E. coli* containing the pET15b vector only. Analysis of reaction products using TLC showed that apigenin, daidzein, genistein, esculetin, kaempferol, naringenin, and quercetin acted as methyl acceptors and produced new products that had different R_f values from those of the parent compounds, while only substrates were detected in negative control experiments. HPLC analysis also showed the appearance of a new peak at a different retention time, when compared to the parent compound. LC/MS analysis of the reaction product showed that the molecular weight of all the new products was increased by 14-DA, suggesting that a methyl group was transferred into the corresponding substrates (data not shown). All of the flavonoids used have 4′-, 5-, and 7-hydroxyl groups, indicating that SaOMT-2 transferred a methyl group into either 4′-, 5-, or 7-hydroxyl group.

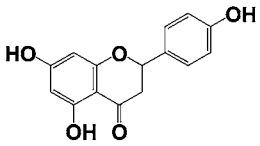
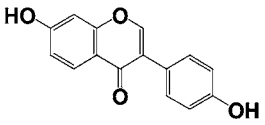
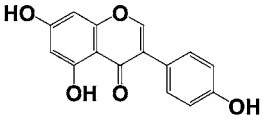
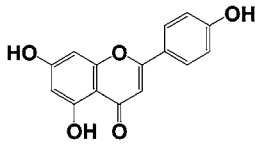
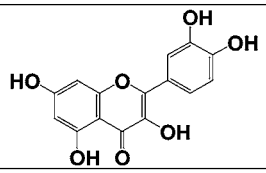
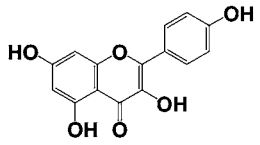
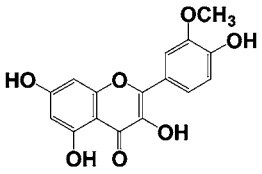
The reaction products of apingenin and naringenin were further analyzed by HPLC. Because 4′- or 7-methylated apingenin and 7-methylated naringenin are commercially available, these compounds were used as authentic compounds. The reaction product from naringenin had the same retention time (19.2 min) and UV spectrum as those of the authentic 7-methylated naringenin, sakuranetin (**Figure 3**). Also, the retention time and UV spectrum of the reaction product formed from apigenin were indistinguishable from those of the authentic 7-methylated compound, genkwanin (data not shown). This suggested that SaOMT-2 was a 7-*O*-methyltransferase.

To further verify the methylated position on the product formed from naringenin by SaOMT-2, the structure of the naringenin reaction product was determined by NMR. The NMR data for naringenin and its reaction product with SaOMT-2 were compared with the previously reported ones (*22*). It turned out that the reaction product was methylated at the C-7 hydroxyl group of naringenin. Apigenin was also reacted with SaOMT-2, and its reaction product was analyzed using NMR spectroscopy. The authentic 4′,5-dihydroxy-7-methoxyflavone could be purchased, and its ^{1}H NMR and ^{13}C NMR data were compared with those of the reaction product of apigenin by SaOMT-2. The ^{1}H and ^{13}C NMR spectra of the reaction product and its authentic sample matched exactly. ^{1}H NMR (DMSO-d_6) δ 7.97 (H-2′, H-6′), 6.94 (H-3′, H-5′), 6.86 (H-3), 6.78 (H-8), 6.38 (H-6), 3.88 (MeO-7); ^{13}C NMR (DMSO-d_6) δ 181.9 (C-4), 165.1 (C-7), 164.1 (C-2), 161.3 (C-4′), 161.2 (C-5), 157.2 (C-9), 128.5 (C-2′, C-6′), 121.1 (C-1′), 116.0 (C-3′, C-5′), 104.7 (C-10), 103.0 (C-3), 98.0 (C-6), 92.7 (C-8), 56.0 (MeO-7). The quercetin reaction product with SaOMT-2 and authentic 7-*O*-methylated quercetin were subject to NMR analysis, and the NMR data were compared. The ^{1}H and ^{13}C NMR spectra of the reaction product and its authentic sample are matched exactly. ^{1}H NMR (DMSO-d_6) δ 7.72 (H-2′), 7.57 (H-6′), 6.89 (H-5′), 6.70 (H-8), 6.35 (H-6), 3.86 (MeO-7); ^{13}C NMR (DMSO-d_6) δ 175.9 (C-4), 164.9 (C-7), 160.3 (C-5), 156.0 (C-9), 147.8 (C-4′), 147.3 (C-2), 145.1 (C-3′), 136.0 (C-3), 121.8 (C-6′), 120.0 (C-1′), 115.6 (C-5′), 115.2 (C-2′), 104.0 (C-10), 97.4 (C-6), 91.9 (C-8), 56.0 (MeO-7). These results showed that SaOMT-2 transferred a methyl group to the C-7 hydroxyl group of flavonoids.

The preference of the substrate was examined by incubating diverse (iso)flavones, such as apigenin, daidzein, geninstein, isorhamnetin, keampferol, and quercetin, and the flavone naringenin with *E. coli* containing *SaOMT-2*. The amounts of the reaction products were analyzed by HPLC peak area. Daidzein was the most effective substrate tested, followed by quercetin, naringenin, and then kaempferol in order. Isorhamnetin, which has a methyl group on the 3′-C of quercetin, was about 50% less methylated than quercetin, itself.

There have been a few 7-*O*-methyltransferases isolated from plants. Isoflavone 7-*O*-methyltransferase (IOMT) from *Medicago sativa* had substrate specificity only for the isoflavones daidzein and genistein. It did not use either flavone apigenin or flavanone naringenin as substrates (*17*). The second is the flavonoid 7-*O*-methyltransferase (F1-OMT) from barley, which showed highest activity toward flavone apigenin, while it was less active toward flavanone (narigenein) and flavonol (kaempferol and quercetin) (*18*). Naringenin 7-*O*-methyltransferase (NOMT) from rice showed high substrate preference toward the flavanone naringenin and the flavones luteolin and apigenin, while it did not have any activity against isoflavones such as daidzein and genitein, and the corresponding gene has not been cloned (*23*). It is likely that *SaOMT-2* is the first 7-*O*-methyltransferase that transfers a methyl group to both flavones and isoflavones and

Table 1. Biotransformation with SaOMT-2 and Its Relative Conversion Rate

	Substrate	Structural formula	Relative conversion rate (%)
Flavanone	Naringenin		100
Isoflavones	Daidzein		125.7
	Genistein		58.7
Flavones	Apigenin		23.4
	Quercetin		119.0
	Kaempferol		99.7
	Isorhamnetin		56.6

[a] Relative conversion rate was calculated with activity against (±) naringenin as 100. [b] Catechol, caffeic acid, formononetin, genkawanin, and skauranetin were tested but were not metabolized. [c] Esculetin showed less than 10% conversion rate.

has a broader substrate range than plant 7-OMTs such as IOMT, F1-OMT, and NOMT (*17*, *18*, *23*).

The in vivo substrate of SaOMT-2 is likely to be a polyketide because it is predicted to be a part of polyketide gene cluster while it could metabolize flavonoids, based on our result. It is generally accepted that flavonoid OMTs from plants are highly specific so that they could not metabolize other classes of compounds but OMT from the micro-organism showed activity toward flavonoids (*14*).

It is not certain that *S. avermitilis* itself could metabolize flavonoids such as naringenin. Cultivation of naringenin with *S. avermitilis* showed only the disappearance of the naringenin, which might be due to the function of nonspecific oxygenases (*24*). Whatever the in vivo substrate of SaOMT-2 is, our results showed the possibility to use microbial genes for modification of flavonoids.

Biotransformation Kinetics of Naringenin with SaOMT-2.

The *E. coli* Sa-2 converted naringenin to sakuranetin, which has an antifungal activity against *Magnaporthe grisea* (*25*). We analyzed the biotransformation kinetics of naringenin with *E. coli* Sa-2. Whereas the amount of naringenin continued to decrease during the time of incubation, there was a corresponding increase in the amount of the reaction products (**Figure 4**). After 15 h of incubation, the production of sakuranetin reached a maximum of 57 μM (approximately 15.3 ppm), whereas the remaining amount of naringenin was about 3 μM. The ED50 value, namely, the amount of sakuranetin required to inhibit germination of *M. grisea* in 50% of the population, of sakuranetin against spore germination of *M. grisiea* was about 15 ppm (*25*), which is approximately the same amount produced by biotransformation.

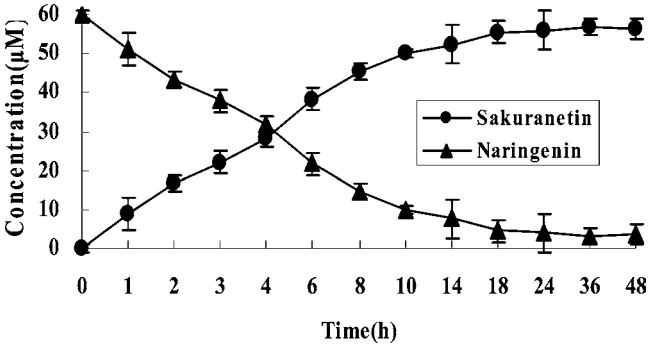


Figure 4. Biotransformation kinetics of naringenin with *E.coli* containing *SaOMT-2* (*E. coli Sa-2*).

So far, sakuranetin was isolated from a UV-treated rice sample with a yield of 10 mg/50 g of UV-treated rice (*25*). On the other hand, about 15 mg of sakuranetin could be produced from a 1-L culture using *E. coli* Sa-2. In addition, the compound which is produced using biotransformation provides regiospecificity and chirality, both of which are not easily achieved by conventional chemical synthesis (*26*). Biotransformation could contribute to saving expensive cofactors, such as *S*-adenosyl-L-methionine, for the methylation reaction and uridine diphosphate glucose (UDP-glucose) for the glycosylation reaction, since these cofactors should be added for the enzymatic reactions. Several studies showed in vitro synthesis of flavonoids using transgenic *E. coli* containing various flavonoid biosynthetic genes. One of these approaches was to reconstruct the flavonoid biosynthetic pathway in *E. coli* using several genes. It was applied for the production of the biosynthesis of naringenin (*27*, *28*) and anthocyanin (*21*). Even though it showed possibilities to produce flavonoids in *E. coli*, it needs to be redefined due to the low yield of final product. The other approach was to use one gene that modifies the structure of flavonoids. This approach resulted in compound with biological value. For example, the production of ponciretin, a potential inhibitor of *Helicobacter pylori* (*29*), was achieved by *E. coli* containing OMT from soybean (*13*), and conversion of naringenin into sakuranetin with SaOMT-2 would be another example. Therefore, the work described in here would extend ways to apply the genome sequence to produce valuable chemicals.

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