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Biochemical characterization of a regiospecific flavonoid 3′-*O*-methyltransferase from orange

Hye Lin Park[1], Seong Hee Bhoo[1], Sang-Won Lee[1*] and Man-Ho Cho[1*]

Abstract

Citrus plants have diverse methoxyflavonoids including, chrysoeriol, isosakuranetin, and nobiletin. In plants, *O*-methyltransferases (OMTs) participate in the methylation of a vast array of secondary metabolites, including flavonoids, phenylpropanoids, and alkaloids. To identify functional OMTs involved in the formation of methoxyflavonoids, orange (*Citrus sinensis*) OMT (CsOMT) genes were retrieved from the Citrus Genome Database. The phylogenetic relationships with functional OMTs suggested that three CsOMTs, CsOMT15, CsOMT16, and CsOMT30, are possible candidates for flavonoid OMTs (FOMTs). These CsOMTs were heterologously expressed in *Escherichia coli*, and their OMT activity was examined with flavonoid substrates. Of the examined CsOMTs, CsOMT16 catalyzed the regiospecific 3′-*O*-methylation of flavonoids to the respective 3′-methoxyflavonoids. A kinetic study demonstrated that CsOMT16 accepts diverse flavonoids as a substrate with a comparable preference. The flavonoids eriodictyol, luteolin, and quercetin were efficiently converted to homoeriodictyol, chrysoeriol, and isorhamnetin by CsOMT16-transformed *E. coli* cells, respectively. These findings suggest that CsOMT16 contributes to the methoxyflavonoid formation in orange and is applicable to the biotechnological production of 3′-methoxyflavonoids.

Keywords Flavonoid 3′-*O*-methyltransferase, Methoxyflavonoid, Orange, Biotransformation

Introduction

Flavonoids are a structurally diverse class of plant secondary metabolites, which are known to have health-beneficial activities [1–5]. The structural diversity of flavonoids comes from the different oxidation statuses of the carbon skeletons and substitutions in their hydroxyl groups. Flavonoids are divided into several subclasses, such as flavanones, flavones, and flavonols, based on the oxidation status of the middle ring [6]. Flavonoid

backbones can be substituted by a variety of functional groups, including methyl, isoprenyl, and glycosyl moieties [7]. The hydroxyl groups of flavonoids are open-modified with one or more methyl groups [7–9]. *O*-Methylation affects the physiochemical properties and biological activities of flavonoids [7, 10–12].

In plants, *O*-methyltransferases (OMTs) are responsible for the methylation of a wide array of secondary metabolites. OMT transfers a methyl group from S-adenosyl L-methionine (SAM) to the hydroxyl group of an acceptor molecule [8, 9]. Two classes of SAM-dependent OMTs are involved in the methylation of phenolic compounds. Class I OMT methylates hydroxycinnamoyl-CoAs and is involved in the formation of monolignols [9]. Class II OMT participates in the methylation of an array of phenolic compounds, including flavonoids and isoflavonoids

*Correspondence:
Sang-Won Lee
swlee6803@khu.ac.kr
Man-Ho Cho
manhocho@khu.ac.kr
[1] Department of Genetics and Biothenology, Kyung Hee University, Yongin 17104, Korea

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[8, 9, 13]. Flavonoid OMTs (FOMTs) accept diverse flavonoid substrates, whereas they typically transfer a methyl group to a specific position of flavonoids [8, 14–16]. Due to their broad substrate selectivity and strict regiospecificity, FOMTs are considered a useful tool for the tailored production of methoxyflavonoids [17, 18].

Citrus plants are a rich source of methoxyflavonoids. Citrus fruit and juices are reported to have diverse methoxyflavonoids, such as hesperetin, isosakuranetin, chrysoeriol, and nobiletin, and their respective glycosides [19–21]. To identify functional FOMTs, orange (*C. sinensis*) OMT genes (CsOMTs) were retrieved from the Citrus Genome Database. Based on the phylogenetic relationships with functional OMTs, three CsOMTs were selected as FOMT candidates and were heterologously expressed in *E. coli as* a His-tag fusion protein. The OMT activity and kinetic properties of the recombinant CsOMT proteins toward flavonoids were examined in the present study. Biotransformation of flavonoids using the *E. coli* transformants harboring CsOMT was also performed for the production of methoxyflavonoids.

Materials and methods
Materials
Flavonoids were purchased from Indofine Chemical Company (Hillsborough, NJ, USA) and Extrasynthese (Genay Cedex, France). The pJET1.2/blunt and pET-28a vectors were bought from Thermo-Fisher Scientific (Waltham, MA, USA) and Novagen (Madison, WI, USA), respectively. Luria–Bertani (LB) broth and isopropyl β-D-thiogalactopyranoside (IPTG) were obtained from Duchefa Biochemie (Haarlem, The Netherlands) and Promega (Madison, WI, USA), respectively. Ni–NTA agarose was bought from Thermo-Fisher Scientific. High-performance liquid chromatography (HPLC) solvents and imidazole were purchased from Samchun Chemicals (Seoul, Korea). Other reagents, including SAM and phenylmethylsulfonyl fluoride (PMSF), were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Phylogenetic analysis and cloning of CsOMTs
Multiple alignments of amino acid sequences of CsOMT and other OMTs were performed with Clustal-W [22]. The evolutionary history of CsOMTs and other OMTs was inferred using the Neighbor-joining method, and the evolutionary distances were computed using the JTT matrix-based method [23, 24]. A phylogenetic tree was drawn in MEGA X [25]. The OMT sequences, CaFOMT (U16794), CaOMT2 (U16793), AtCOMT (NP_200227), PtOMT1 (X62096), MsCOMT (M63853), IEMT (U86760), SlOMT3 (AK325603), TaCM (EF413031), TaOMT-2 (DQ223971), PvCOMT (HQ645965), OsCOMT (XP_015650053), MpOMT3 (AY337460), MpOMT4

(AY337461), ObFOMT5 (AFU50299), MpOMT1 (AY337457), ShMOMT2 (JF499657), CrOMT6 (AY343489), GeHI4OMT (AB091684), LjHI4OMT (AB091686), MtIOMT5 (AY942158), MtIOMT6 (DQ419913), GeD7OMT (AB091685), MtIOMT1 (AY942159), MsIOMT (U97125), IOMT (AAC49927), were used to draw phylogenetic tree (accession numbers are in parentheses).

First-strand cDNA was synthesized from the total RNA extracted from orange peel by the procedures described previously [16]. The CsOMT16 (accession number; OR786463) gene was amplified by polymerase chain reaction (PCR) from the first-strand cDNA. The primers used were 5′-GG<u>CATATG</u>GGTTCAACCAGTTCA GAA-3′ and 5′-CC<u>GGATCC</u>TCAAGCACTCTTGAG AAATTCC-3′ (NdeI and BamHI sites are underlined, respectively). The PCR product was cloned into the pJET1.2/blunt vector. After sequence confirmation, the CsOMT16 gene was inserted into the pET-28a vector. Even though it was attempted several times, the CsOMT15 and CsOMT30 genes could not amplify from the first-strand cDNA of orange. Therefore, the synthetic CsOMT15 and CsOMT30 genes individually cloned in the pET-28b vector were obtained from Bionics (Seoul, Korea). For the heterologous expression of CsOMTs, the resulting constructs were individually transformed into *E. coli* BL21 (DE3) and Rosetta2 (DE3) cells.

Expression and purification of recombinant CsOMTs
The *E. coli* transformants harboring each CsOMT/pET-28 construct were inoculated into LB medium supplemented with kanamycin (25 μg/mL) and grown at 37 °C until the cell population reached an OD_{600} of ~0.8. Different concentrations of IPTG were added to the cultures, and the cells were then grown at various temperatures to induce recombinant CsOMT expression. After overnight induction, the cells were harvested by centrifugation and stored in a freezer until use. The cells were resuspended in phosphate-buffered saline (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) supplemented with lysozyme (1 mg/mL) and PMSF (1 mM), and the resulting cell suspensions were then sonicated to break cells. After the removal of cell debris, the crude protein extract was applied to a Ni–NTA agarose column. The recombinant CsOMT proteins were eluted with different concentrations of imidazole in 50 mM Tris–HCl buffer (pH 8.0, 300 mM NaCl). Protein purification was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel.

FOMT assay and kinetic analysis
FOMT reactions of recombinant CsOMTs were carried out in 20 mM Tris–HCl buffer (pH 7.5) containing

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50 μM flavonoid substrate and 100 μM SAM. The reaction was initiated by the individual addition of recombinant CsOMT protein. After incubation of the reaction mixtures at 30 °C for 30 min, a one-tenth volume of 5 N HCl was added to stop the reaction. The reaction mixtures were extracted twice with ethyl acetate, and the resulting extracts were then dried *in vacuo*. The residues were redissolved in dimethyl sulfoxide and analyzed by reversed-phase HPLC equipped with a Sunfire C$_{18}$ column (Waters, Milford, MA, USA) using a linear gradient of 25–60% acetonitrile in 3% acetic acid–water for 25 min with detection at 280 nm. Liquid chromatography-mass spectrometry (LC–MS) analysis of CsOMT reaction mixtures and authentic 3'-methoxyflavonoids was carried out with an Agilent 6410 Triple Quadrupole LC–MS System (Agilent Technologies, Santa Clara, CA) to identify the reaction products. Mass spectra were obtained with the ion source of negative-mode electrospray ionization.

Kinetic analysis of CsOMT reactions was performed with different concentrations of flavonoids. Eriodictyol and luteolin concentrations for kinetic analysis were 0.5–10 μM. The concentrations of quercetin and rhamnetin were 0.5–50 μM.

Biotransformation of flavonoids using CsOMT16-transformed *E. coli*

The *E. coli* cells bearing the CsOMT16/pET-28a construct were grown in LB medium supplemented with kanamycin (25 μg/mL) by the above-described method. The *E. coli* culture was added with 0.1 mM IPTG and followed by 4 h incubation at 25 °C to induce CsOMT16 expression. After the induction, the culture was centrifuged, and the medium was discarded. The cell pellet was resuspended in the same volume of fresh LB medium supplemented with kanamycin (25 μg/mL). Each flavonoid substrate was added to the cell suspension at a concentration of 50 μM and further incubated at 25 °C. An aliquot of the culture was harvested at the selected time points and centrifuged to remove the cells. Each cell-free medium was extracted twice with ethyl acetate. The resulting extracts were dried in vacuo. The residues were individually redissolved in a small volume of dimethyl sulfoxide and analyzed by reversed-phase HPLC using the method described above.

Results and discussion

Phylogenetic analysis and molecular characterization of CsOMTs

Plant genomes contain several dozens of class II OMT genes, of which a few encode biochemically functional OMTs [14, 26, 27]. More than a hundred CsOMTs were retrieved from the Citrus Genome Database (https://

www.citrusgenomedb.org/) by searching for the gene ontology term *O-methyltransferase activity*. Class II OMTs are known to have an *O*-methyltransferase domain (InterPro ID: IPR001077) and a dimerisation domain of plant methyltransferase (InterPro ID: IPR012967) [28–30]. A domain search of CsOMTs using the InterPro search tool (https://www.ebi.ac.uk/interpro/) revealed that 32 members harbor both *O*-methyltransferase and dimerisation domains, designating them as CsOMT1-CsOMT32 [31].

A phylogenetic analysis of functional OMTs showed that Class II OMTs are divided into two categories, FOMTs and isoflavonoid OMTs (IFOMTs), based on their substrate specificity (Fig. 1 and Additional file 1: Fig. S1). Caffeic acid OMT (COMT), which catalyzes the methylation of hydroxycinnamic acids such as caffeic and 5-hydroxyferulic acids, is a representative member of class II OMT [8, 9]. *Arabidopsis thaliana* COMT (AtCOMT), *Medicago sativa* COMT (MsCOMT), *Panicum virgatum* COMT (PvCOMT), and *Oryza sativa* COMT (OsCOMT) were categorized as FOMTs (Fig. 1 and Additional file 1: Fig. S1). IFOMTs are known to be restricted in legumes [32, 33]. Within the FOMT category, COMTs were closely related to several FOMTs, including *Chrysoplenium americanum* OMTs (CaOMT2 and CaFOMT), *Solanum lycopersicum* OMT3 (SlOMT3), and *Mentha piperita* OMT3 (MpOMT3), being designated as group I (Fig. 1). These FOMTs have been reported to catalyze the methylation of the 3′-OH and/or 5′-OH of flavonoids [14, 34, 35]. It is well agreed that the 3′-OH and 5′-OH of flavonoids are derived from the 3-OH and 5-OH of hydroxycinnamic acids that are methylated by COMTs [30]. CsOMT15 and CsOMT16 showed a close relationship with the group I FOMTs. FOMTs such as *Ocimum basilicum* FOMT5 (ObFOMT5), *M. piperita* OMT4 (MpOMT4), and *Catharanthus roseus* OMT6 (CrOMT6) form another group (group II) separated from group I, which have been identified as flavonoid 7- or 4'-OMTs (Fig. 1) [36, 37]. CsOMT30 was shown to belong to the group II FOMT (Fig. 1).

Open reading frames of CsOMT15, CsOMT16, and CsOMT30 are 1065–1101 nucleotides, encoding polypeptide chains of 354–366 amino acids long (Table 1), which are comparable with typical class II OMT [8, 9, 13]. The His, Glu/Asp, and Glu residues involved in the catalysis of Class II OMT were identified from the structural studies of chalcone OMT (ChOMT), isoflavone OMT (IOMT), and MsCOMT from alfalfa [29, 30]. Three catalytic residues were conserved in CsOMT15 (His267, Asp295, and Asp327), CsOMT16 (His270, Asp298, and Asp330), and CsOMT30 (His258, Asp287, and Asp320) (Fig. 2). It has been reported that

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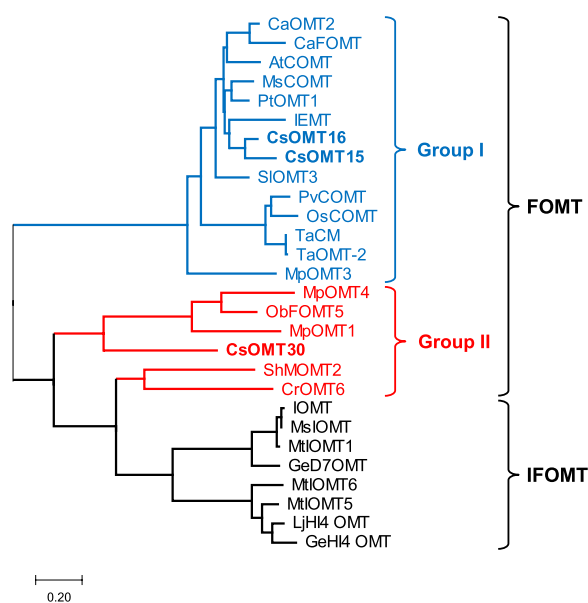
Fig. 1 Phylogenetic relationships between the selected CsOMTs and other functional OMTs. The evolutionary history of CsOMTs and other OMTs was analyzed using the Neighbor-joining method. The scale bar represents the evolutionary distances computed using the JTT matrix-based method. A phylogenetic tree was drawn in MEGA X (https://www.megasoftware.net/). The OMT sequences used to draw phylogenetic tree were CaFOMT and CaOMT2 from *Chrysoplenium americanum*, AtCOMT from *Arabidopsis thaliana*, PtOMT1 from *Populus tremuloides*, MsCOMT, MsIOMT and IOMT from *Medicago sativa*, IEMT from *Clarkia breweri*, SlOMT3 from *Solanum lycopersicum*, TaCM and TaOMT-2) from *Triticum aestivum*, PvCOMT from *Panicum virgatum*, OsCOMT from *Oryza sativa*, MpOMT1, MpOMT3 and MpOMT4 from *Mentha piperita*, ObFOMT5 from *Ocimum basilicum*, ShMOMT2 from *Solanum habrochaites*, CrOMT6 from *Catharanthus roseus*, GeHl4OMT and GeD7OMT from *Glycyrrhiza echinata*, LjHl4OMT from *Lotus japonicas*, and MtlOMT1, MtlOMT5 and MtlOMT6 from *Medicago truncatula*

Class II OMTs have SAM binding motifs (SAM-A, B, and C) and COMT motifs (COMT-I, J, K, and L) [8, 9, 13]. Like the group I OMTs (AtCOMT and SlOMT3), CsOMT15 and CsOMT16 have highly conserved SAM-binding and COMT motifs (Fig. 2). A multiple alignment

of amino acid sequences showed that group II members CsOMT30 and ObFOMT5 have the signature motifs with some variations (Fig. 2), likely reflecting different regiospecificity. The consensus sequence of the COMT-L motif suggested previously was GGKERTXXEFLA [13]. Our alignment result showed that the last two residues in the COMT-L motif are highly variable (Fig. 2). The two residues were also reported to vary in *Perilla frutescens* OMT3 (PfOMT3), OsNOMT, and ChOMT [16]. Therefore, the COMT-L motif is better revised as GGKERTXXEF. The phylogenetic relationships with other functional OMTs and the conservation of the signature motifs suggest that CsOMT15, CsOMT16, and CsOMT30 are putative candidates of FOMTs in orange.

Heterologous expression and purification of CsOMTs

The cDNAs of CsOMT15, CsOMT16, and CsOMT30 were cloned into either the pET-28a or pET-28b vectors. The resulting constructs were individually transformed into *E. coli* BL21 and Rosetta2 cells for heterologous expression of CsOMT15, CsOMT16, and CsOMT30 as a His-tag fusion protein. CsOMT16 and CsOMT30 were successfully expressed in *E. coli* BL21 cells at 25 °C of growth temperature by 0.1 mM and 0.5 mM IPTG, respectively. Although the expression was attempted under various induction conditions, CsOMT15 was not expressed as a soluble form in both *E. coli* BL21 and Rosetta2 cells. Recombinant CsOMT16 and CsOMT30 proteins were purified by Ni^{2+} affinity chromatography to examine their biochemical properties (Fig. 3). An SDS-PAGE analysis showed that the molecular sizes of the purified CsOMTs were consistent with the theoretical molecular masses of the His-tagged CsOMT16 and CsOMT30 proteins (Fig. 3).

### FOMT activity and regiospecificity of CsOMTs

To identify biochemically functional CsOMTs, SAM-dependent FOMT activity of recombinant CsOMT16 and CsOMT30 proteins was examined with various subclasses of flavonoid substrates, including flavanones, flavones, and flavonols (Fig. 4). An HPLC analysis of the CsOMT reactions showed that CsOMT16 consumed

Table 1 Molecular properties of CsOMT15, CsOM16, and CsOMT30

Name	Gene ID[a]	ORF (bp)	Protein length (aa)	Protein domain (amino acid position)	
				Dimerisation (IPR012967[b])	*O*-Methyltransferase (IPR001077[b])
CsOMT15	Cs5g13560	1092	363	32–83	138–343
CsOMT16	Cs5g16290	1101	366	35–86	141–346
CsOMT30	Cs8g02130	1065	354	33–76	127–336

[a] Gene ID obtained from *Citrus sinensis* Valencia genome in the Citrus Genome Database

[b] InterPro domain ID
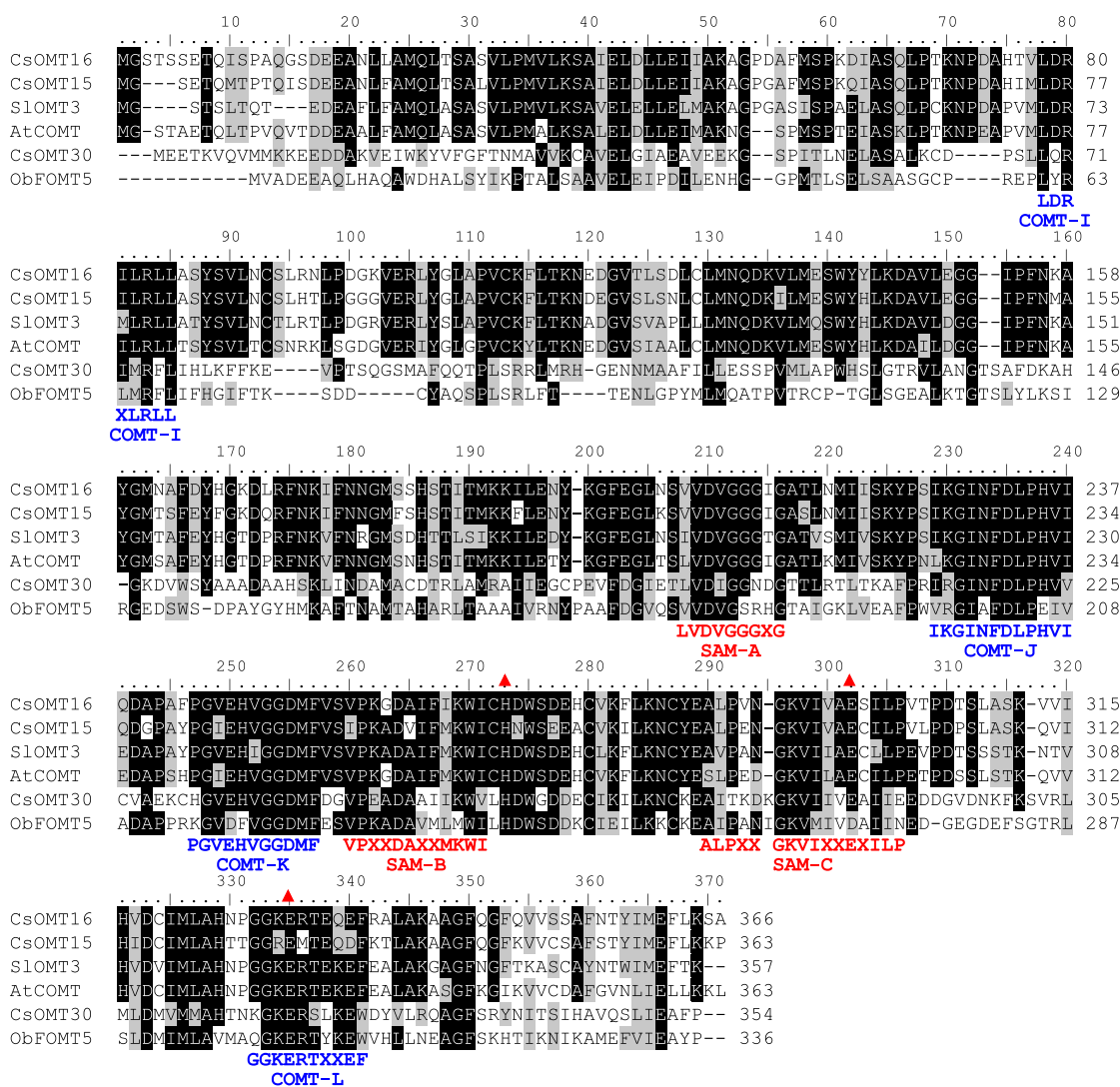
Fig. 2 The conserved signature motifs of class II OMT in CsOMTs, SlOMT3, AtCOMT, and ObFOMT5. The amino acid sequences of CsOMTs were aligned with those of class II OMT SlOMT3, AtCOMT, and ObFOMT5 using Clustal-W. The SAM binding motifs and the COMT motifs were indicated under the aligned sequences with their consensus sequences (X indicates any amino acid). Red triangles indicate the catalytic residues of class II OMT. Identical and similar amino acids are shaded in black and grey, respectively

diverse flavonoids, such as eriodictyol, luteolin, and quercetin, and yielded the reaction products (Fig. 5). As expected, the products of the CsOMT16 reactions showed 14 daltons higher mass than their substrates in the mass spectra, indicating that CsOMT16 catalyzes the SAM-dependent methylation of flavonoids (Additional file 1: Fig. S2). Unlike the CsOMT16 reactions, no product peak was detected in the CsOMT30 reactions with flavonoid substrates. This result indicates that CsOMT16 is a biochemically functional FOMT and CsOMT30 has no FOMT activity. CsOMT16 accepted diverse substrates of different flavonoid backbones, although its FOMT activity varied considerably depending on flavonoids (Table 2). This property is well consistent with the broad substrate specificity of other FOMTs [14–18, 26, 27].

It has been known that FOMTs catalyze the regiospecific *O*-methylation of flavonoids [14–18, 27]. CsOMT16 used most flavonoids examined as a substrate. However, no product was detected in CsOMT16 reactions with naringenin, apigenin, and
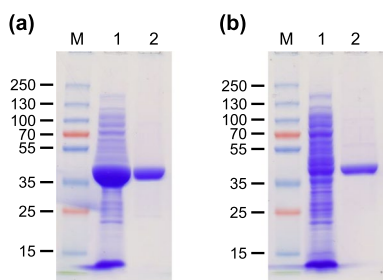
Fig. 3 Purification of recombinant CsOMT16 (**a**) and CsOMT30 (**b**) proteins expressed in *E. coli*. M, size marker; 1, crude extract from IPTG-induced culture of the *E. coli* transformants; 2, affinity-purified recombinant CsOMT protein

kaempferol (Table 2). These flavonoids commonly have no 3'-OH in their backbones (Fig. 4), suggesting that CsOMT16 requires an OH group at the 3'-position. Indeed, the reaction products from eriodictyol, luteolin, and quercetin showed identical retention times with the authentic 3'-methoxyflavonoids, homoeriodictyol, chrysoeriol, and isorhamnetin, respectively (Fig. 5). A LC–MS analysis also showed that the fragmentation pattern of the products of the CsOMT16 reactions was almost identical with that of the corresponding 3'-methoxyflavonoids (Additional file 1: Fig. S2), indicating that CsOMT16
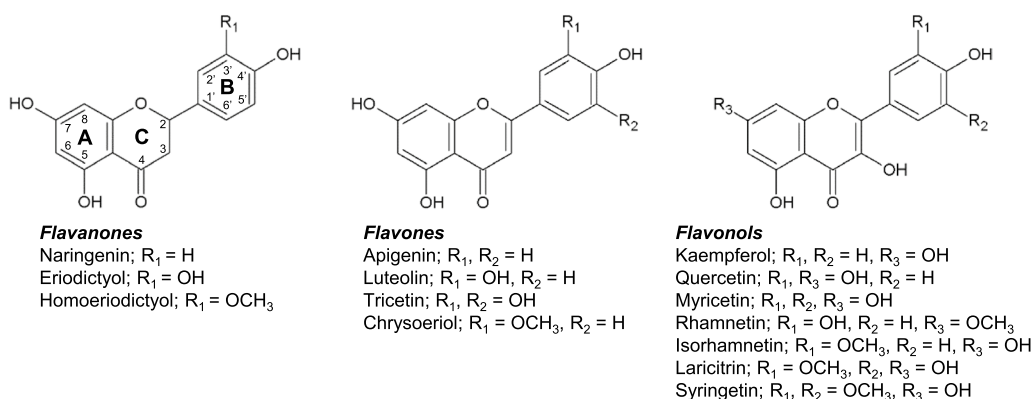
Fig. 4 Three subclasses of flavonoids examined in this study
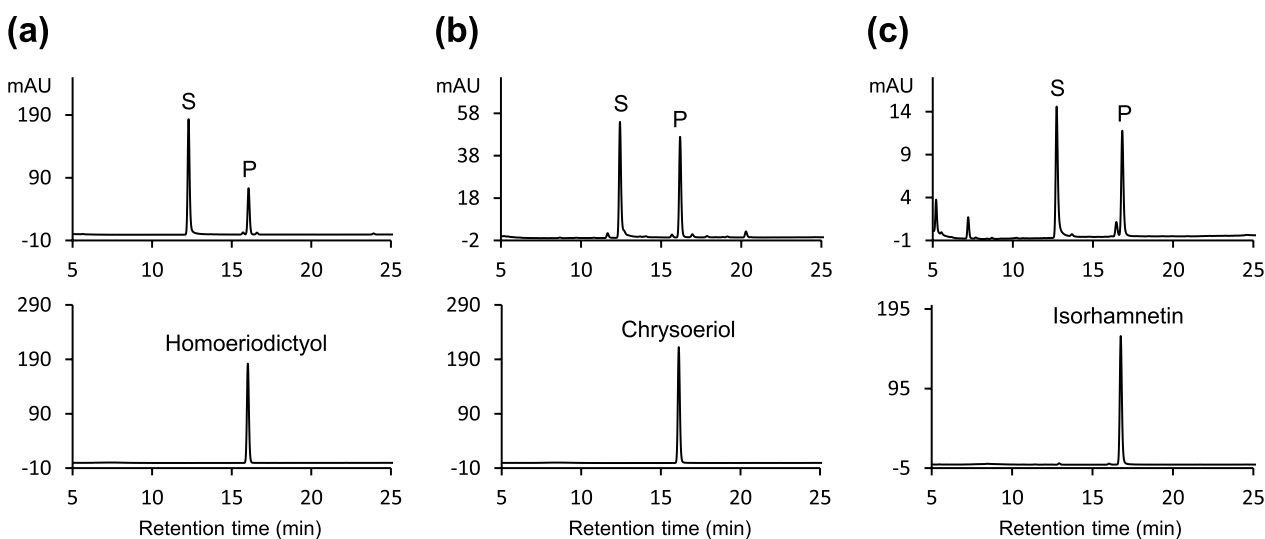
Fig. 5 HPLC analysis of the CsOMT16 reactions with flavonoid substrates. The upper panels are HPLC chromatograms of the CsOMT16 reaction mixtures with eriodictyol (**a**), luteolin (**b**), and quercetin (**c**). Lower panels are chromatograms of authentic 3'-methoxyflavonoids, homoeriodictyol, chrysoeriol, and isorhamnetin. *S* substrate, *P* reaction product

Table 2 Substrate preference of recombinant CsOMT16 expressed in *E. coli*

Substrate	Relative activity (%)[a]	Reaction product
Flavanone		
Naringenin	—[b]	
Eriodictyol	33.89	Homoeriodictyol
Flavone		
Apigenin	—	
Luteolin	75.61	Chrysoeriol
Tricetin	+[c]	
Flavonol		
Kaempferol	—	
Quercetin	100	Isorhamnetin
Rhamnetin	6.74	Rhamnazin
Myricetin	+	Laricitrin, syringetin

[a] The OMT activity toward flavonoids respective to that toward quercetin at a substrate concentration of 50 μM

[b] Minus signs indicate that no product was detected in the HPLC analysis

[c] Plus signs represent that the flavonoids tested were used as a substrate that yielded a product peak in the HPLC analysis. The relative activities of the substrates were not determined

Table 3 Kinetic parameters of recombinant CsOMT16[a]

Substrate	K_m (μM)	V_{max} (pmol min^{-1} μg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Eriodictyol	2.62 ± 0.75	32.25 ± 3.81	1.36	8.66×10^3
Luteolin	1.17 ± 0.09	60.62 ± 9.28	2.55	3.65×10^4
Quercetin	4.52 ± 0.81	89.33 ± 5.36	3.76	1.39×10^4
Rhamnetin	0.25 ± 0.05	10.34 ± 0.32	0.44	2.91×10^4

[a] The results represent the mean and standard deviation from three independent experiments

catalyzes the 3'-*O*-methylation of flavonoids. Even though there are a few exceptions, flavonoid 3′-OMTs commonly catalyze methylation at the 3′- and 5′-positions of substrates because the 3′- and 5′-positions of flavonoids are chemically equivalent [14, 38, 39]. Similarly, CsOMT16 catalyzed both 3'- and 5'-*O*-methylation of flavonoids, which contain 3'- and 5'-OH groups. Myricetin and tricetin harboring 3'- and 5'-OH groups were used by CsOMT16 and yielded the dimethylated products (Table 2). A LC–MS analysis of the CsOMT16 reaction with myricetin and laricitrin (3'-methylmyricetin) revealed that the fragmentation pattern of the methylated products of these substrates was almost identical with that of syringetin (3',5'-dimethylmyricetin), indicating that CsOMT16 can catalyze an additional 5'-*O*-methylation of flavonoids when the 5'-OH is available. These results demonstrate that CsOMT16 is a FOMT catalyzing the 3'-*O*-methylation of flavonoids and likely contributes to orange having diverse methoxyflavonoids.

Substrate preferences of CsOMT16

Like other FOMTs, CsOMT16 methylates diverse flavonoids to different extents. Under saturated conditions of substrates, CsOMT16 showed the strongest OMT activity toward quercetin among the examined flavonoids (Table 2). In the same condition, the activity of CsOMT16 for rhamnetin was about 15-fold lower than that for quercetin. CsOMT16 exhibited moderate OMT

activity for eriodictyol and luteolin relative to those for quercetin and rhamnetin (Table 2).

To further elucidate the biochemical properties of CsOMT16, the kinetic parameters of recombinant CsOMT16 toward the selected flavonoid substrates, eriodictyol, luteolin, quercetin, and rhamnetin, were determined. In agreement with its relative activity, CsOMT16 has the highest V_{max} and k_{cat} values of 89.33 pmol min^{-1} ug^{-1} and 3.76 min^{-1} for quercetin, respectively (Table 3). The V_{max} value for rhamnetin was the lowest among those for the examined substrates. The V_{max} values of CsOMT16 for eriodictyol and luteolin were 32.25 pmol min^{-1} ug^{-1} and 60.62 pmol min^{-1} ug^{-1}, respectively. CsOMT16 showed comparable binding affinity to eriodictyol, luteolin, and quercetin, with K_m values of 2.62, 1.17, and 4.52 μM, respectively (Table 3). CsOMT16 had the lowest K_m value of 0.25 μM for rhamnetin. Although its V_{max} and K_m values were considerably different, CsOMT16 exhibited comparable k_{cat}/K_m values to the flavonoids examined, ranging from 8.66×10^3 to 3.65×10^4 M^{-1} s^{-1} (Table 3). The kinetic analysis demonstrates that CsOMT16 accepts a broad range of flavonoid substrates with a minor difference in catalytic activity.

Production of 3′-methoxyflavonoids using CsOMT16-transformed E. coli

Because of their substrate-, regio-, and stereo-specificities, biocatalysts such as enzymes and whole-cell microorganisms have been applied to selectively modify synthetic and natural compounds [40–43]. Several FOMTs were reported to be attempted in the regiospecific methylation of flavonoids by biotransformation. *E. coli* cells expressing the flavonoid 4′-OMT from soybeans were used to produce pociretin, an anti-microbial flavonoid against *Helicobacter pylori*, from naringenin [44]. The 7-methoxyflavonoids sakuranetin and rhamnocitrin were produced from naringenin and kaempferol, respectively, by *E. coli* cells harboring PfOMT3, a flavonoid 7-OMT [16].

To evaluate the application of CsOMT16 in the biotechnological production of 3'-methoxyflavonoids,

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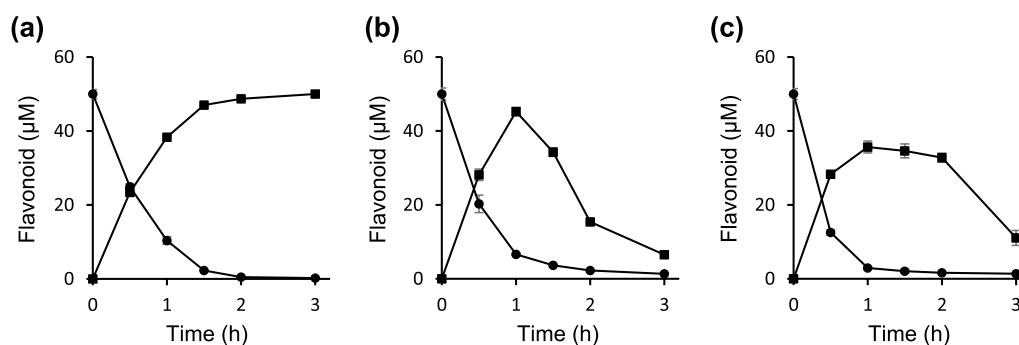
Fig. 6 Biotransformation of flavonoids using CsOMT16-transformed *E. coli* cells. Bioconversion of the flavonoid substrates (●) eriodictyol (**a**), luteolin (**b**), and quercetin (**c**) to their respective 3′-*O*-methylated products (■) homoeriodictyol, chrysoeriol, and isorhamnetin, respectively, by the *E. coli* transformants bearing CsOMT16. The results represent the mean and standard deviation from two independent experiments

the biotransformation of eriodictyol, luteolin, and quercetin was performed with *E. coli* transformants bearing CsOMT16. The culture of the transformed *E. coli* was added with IPTG to induce CsOMT16 expression. After the induction of CsOMT16, the flavonoids (50 μM) were individually added to the culture, and then the production of the respective 3'-methoxyflavonoids was monitored. Eriodictyol was almost consumed by the *E. coli* cells within 2 h and was converted to an equivalent amount of homoeriodictyol (Fig. 6a). In parallel with the substrate preference of CsOMT16, the bioconversion of luteolin and quercetin by the CsOMT16-transformed *E. coli* was faster than that of eriodictyol. The bioconversion yield of eriodictyol homoeriodictyol in the first 30 min of biotransformation was 46.8%, and that of luteolin and quercetin was 56.3 and 56.6%, respectively. The production of the respective 3'-*O*-methylated products from luteolin and quercetin was reached at a maximum level after 1 h of biotransformation and was then declined (Fig. 6b, c). The maximum biotransformation yield of chrysoeriol and isorhamnetin from luteolin and quercetin was 90.5 and 71.3%, respectively, at the substrate concentration of 50 μM.

Methoxyflavonoids have valuable biological activities and have thus been considered functional agents in the food and pharmaceutical industries [11, 12, 45–48]. 3'-Methoxyflavonoids such as homoeriodictyol, chrysoeriol, and isorhamnetin have been reported to have anti-cancer, anti-inflammatory, and anti-oxidation activities [4, 5, 46, 49, 50]. Homoeriodictyol has bitter masking effects and is thus applicable in food and pharmaceuticals as a taste modifier [51]. Homoeriodictyol and chrysoeriol exhibit anti-microbial activities against bacteria and fungi [5, 45]. For the application of FOMTs in the biotechnological production of valuable methoxyflavonoids, their biochemical characteristics,

including substrate preferences, regiospecificity, and kinetic properties, need to be elucidated. In this study, CsOMT16 was isolated from orange and identified as a flavonoid 3'-OMT. Biotransformation using *E. coli* cells harboring CsOMT16 also showed the successful production of homoeriodictyol, chrysoeriol, and isorhamnetin from eriodictyol, luteolin, and quercetin, respectively. These results indicate that whole-cell microorganisms bearing CsOMT16 are applicable for the biotechnological production of 3'-methoxyflavonoids.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article [and its Additional files].

Declarations

Competing interests
The authors declare that they have no competing interests.

References
1. Greenwald P (2004) Clinical trials in cancer prevention: current results and prospectives for the future. J Nutr 134:3507S-3512S
2. Hou DX, Fujii M, Terahara N, Yoshimoto M (2004) Molecular mechanisms behind the chemopreventive effects of anthocyanidins. J Biomed Biotechnol 2004:321–325
3. Popiolkiewicz J, Polkowski K, Skierski JS, Mazurek AP (2005) In vitro toxicity evaluation in the development of new anticancer drugs-genistein glycosides. Cancer Lett 229:67–75
4. Gong G, Guan YY, Zhang ZL, Rahman K, Wang SJ, Zhou S, Luan X, Zhang H (2020) Isorhamnetin: a review of pharmacological effects. Biomed Pharmacother 128:110301
5. Aboulaghras S, Sahib N, Bakrim S, Benali T, Charfi S, Guaouguaou FE, El Omari N, Gallo M, Montesano D, Zengin G, Taghzouti K, Bouyahya A (2022) Health benefits and pharmacological aspects of chrysoeriol. Pharmaceuticals 15:973
6. Kumar S, Pandey AK (2013) Chemistry and biological activities of flavonoids: an overview. Sci World J 2013:162750
7. Williams CA, Grayer RJ (2004) Anthocyanins and other flavonoids. Nat Prod Rep 21:539–573
8. Ibrahim RK, Bruneau A, Bantignies B (1998) Plant *O*-methyltransferases: molecular analysis, common signature and classification. Plant Mol Biol 36:1–10
9. Lam KC, Ibrahim RK, Behdad B, Dayanandan S (2007) Structure, function, and evolution of plant *O*-methyltransferases. Genome 50:1001–1013
10. Ibrahim RK, De Luka V, Khouri H, Latchinian L, Brisson L, Charest PM (1987) Enzymology and compartmentation of polymethylated flavonol glucosides in *Chrysosplenium americanum*. Phytochemistry 26:1237–1245
11. Aida Y, Tamogami S, Kodama O, Tsukiboshi T (1996) Synthesis of 7-methoxyapigeninidin and its fungicidal activity against *Gloeocercospora sorghi*. Biosci Biotech Biochem 60:1495–1496
12. Zhang L, Kong Y, Wu D, Zhang H, Wu J, Chen J, Ding J, Hu L, Jiang H, Shen X (2008) Three flavonoids targeting the β-hydroxyacyl-acyl carrier protein dehydratase from *Helicobacter pylori*: Crystal structure characterization with enzymatic inhibition assay. Protein Sci 17:1971–1978
13. Joshi C, Chiang VL (1998) Conserved sequence motifs in plant S-adenosyl-L-methionine-dependent methyltransferases. Plant Mol Biol 37:663–674
14. Cho MH, Park HL, Park JH, Lee SW, Bhoo SH, Hahn TR (2012) Characterization of a regiospecific flavonoid 3′/5′-*O*-methyltransferase from tomato and its application in flavonoid biotransformation. J Korean Soc Appl Biol Chem 55:749–755
15. Shimizu T, Lin F, Hasegawa M, Okada K, Nojiri H, Yamane H (2012) Purification and identification of naringenin 7-*O*-methyltransferase, a key enzyme in biosynthesis of flavonoid phytoalexin sakuranetin in rice. J Biol Chem 287:19315–19325
16. Park HL, Lee JC, Lee K, Lee JM, Nam HJ, Bhoo SH, Lee TH, Lee SW, Cho MH (2020) Biochemical characterization of a flavonoid *O*-methyltransferase from perilla leaves and its application in 7-methoxyflavonoid production. Molecules 25:4455
17. Kim BG, Sung SH, Chong Y, Lim Y, Ahn JH (2010) Plant flavonoid *O*-methyltransferases: substrate specificity and application. J Plant Biol 53:321–329
18. Lee D, Park HL, Lee SW, Bhoo SH, Cho MH (2017) Biotechnological production of dimethoxyflavonoids using a fusion flavonoid *O*-methyltransferase possessing both 3′- and 7-*O*-methyltransferase activities. J Nat Prod 80:1467–1474
19. Gattuso G, Barreca D, Gargiulli C, Leuzzi U, Caristi C (2007) Flavonoid composition of citrus juices. Molecules 12:1641–1673
20. Jeong SW, Kim HG, Park S, Lee JH, Kim YH, Kim GS, Jin JS, Kwak YS, Huh MR, Lee JE, Song Y, Shin SC (2014) Variation in flavonoid levels in *Citrus benikoji* Hort. ex. Tan. infected by *Colletotrichum gloeosporioides*. Food Chem 148:284–288
21. Barreca D, Gattuso G, Bellocco E, Calderaro A, Trombetta D, Smeriglio A, Laganà G, Daglia M, Meneghini S, Nabavi SM (2017) Flavanones: citrus phytochemical with health-promoting properties. BioFactors 43:495–506
22. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
23. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
24. Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8:275–282
25. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549
26. Itoh N, Iwata C, Toda H (2016) Molecular cloning and characterization of a flavonoid-*O*-methyltransferase with broad substrate specificity and regioselectivity from *Citrus depressa*. BMC Plant Biol 16:180
27. Liu H, Xu RX, Gao S, Cheng AX (2017) The functional characterization of a site-specific apigenin 4′-*O*-methyltransferase synthesized by the liverwort species *Plagiochasma appendiculatum*. Molecules 22:769
28. Keller NP, Dischinger HC Jr, Bhatnagar D, Cleveland TE, Ullah AH (1993) Purification of a 40-kilodalton methyltransferase active in the aflatoxin biosynthetic pathway. Appl Environ Microbiol 59:479–484
29. Zubieta C, He XZ, Dixon RA, Noel JP (2001) Structures of two natural product methyltransferases reveal the basis for substrate specificity in plant *O*-methyltransferases. Nat Struct Biol 3:271–279
30. Zubieta C, Kota P, Ferrer JL, Dixon RA, Noel JP (2022) Structural basis for the modulation of lignin monomer methylation by caffeic acid/5-hydroxyferulic acid 3/5-*O*-methyltransferase. Plant Cell 14:1265–1277
31. Paysan-Lafosse T, Blum M, Chuguransky S, Grego T, Pinto BL, Salazar GA, Bileschi ML, Bork P, Bridge A, Colwell L, Gough J, Haft DH, Letunić I, Marchler-Bauer A, Mi H, Natale DA, Orengo CA, Pandurangan AP, Rivoire C, Sigrist CJA, Sillitoe I, Thanki N, Thomas PD, Tosatto SCE, Wu CH, Bateman A (2023) InterPro in 2022. Nucleic Acids Res 51:D418–D427
32. He XZ, Reddy JT, Dixon RA (1998) Stress responses in alfalfa (*Medicago sativa* L). XXII. cDNA cloning and characterization of an elicitor-inducible isoflavone 7-*O*-methyltransferase. Plant Mol Biol 36:43–54
33. Uchida K, Sawada Y, Ochiai K, Sato M, Inaba J, Hirai MY (2020) Identification of a unique type of isoflavone *O*-methyltransferase, GmIOMT1, based on multi-omics analysis of soybean under biotic stress. Plant Cell Physiol 61:1974–1985
34. Gauthier A, Gulick PJ, Ibrahim RK (1998) Characterization of two cDNA clones which encode *O*-methyltransferases for the methylation of both flavonoid and phenylpropanoid compounds. Arch Biochem Biophys 351:243–249
35. Willits MG, Giovanni M, Prata RTN, Kramer CM, De Luca V, Steffens JC, Graser G (2004) Bio-fermentation of modified flavonoids: an example of in vivo diversification of secondary metabolites. Phytochemistry 65:31–41
36. Schröder G, Wehinger E, Lukačin R, Wellmann F, Seefelder W, Schwab W, Schröder J (2004) Flavonoid methylation: a novel 4′-*O*-methyltransferase from *Catharanthus roseus*, and evidence that partially methylated

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flavanones are substrates of four different flavonoid dioxygenases. Phytochemistry 65:1085–1094

37. Berim A, Hyatt DC, Gang DR (2012) A set of regioselective *O*-methyltransferases gives rise to the complex pattern of methoxylated flavones in sweet basil. Plant Physiol 160:1052–1069

38. Cacace S, Schröder G, Wehinger E, Strack D, Schmidt J, Schröder J (2003) A flavonol *O*-methyltransferase from *Cathranthus roseus* performing two sequential methylations. Phytochemistry 62:127–137

39. Schmidt A, Li C, Shi F, Jones AD, Pichersky E (2011) Polymethylated myricetin in trichomes of the wild tomato species *Solanum habrochaites* and characterization of trichome-specific 3′/5′- and 7/4′-myricetin *O*-methyltransferases. Plant Physiol 155:1999–2009

40. Azerad R (2001) Chemical biotechnology: better enzymes for green chemistry. Curr Opin Biotechnol 12:533–534

41. Patel RN (2001) Biocatalytic synthesis of intermediates for the synthesis of chiral drug substances. Curr Opin Biotechnol 12:587–604

42. Fowler ZL, Koffas MAG (2009) Biosynthesis and biotechnological production of flavanones: current state and perspectives. Appl Microbiol Biotechnol 83:799–808

43. Kang JY, Park WJ, Yoon Y, Kim BG (2022) Production of isoquercitrin from quercetin by biotransformation using *Bascillus*. sp CSQ10 isolated from Camellia sinensis cultivation soils. Appl Biol Chem 65:59

44. Kim DH, Kim BG, Lee Y, Ryu JY, Lim Y, Hur HG, Ahn JH (2005) Regiospecific methylation of naringenin to ponciretin by soybean *O*-methyltransferase expressed in *Escherichia coli*. J Biotechnol 119:155–162

45. Garo E, Maillard M, Antus S, Mavi S, Hostettmann K (1996) Five flavans from *Mariscus psilostachys*. Phytochemistry 43:1265–1269

46. Doostdar H, Burke MD, Mayer RT (2000) Bioflavonoids: selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1. Toxicology 144:31–38

47. Suomela JP, Ahotupa M, Yang B, Vasankari T, Kallio H (2006) Absorption of flavonols derived from sea buckthorn (*Hippophaë rhamnoides* L.) and their effect on emerging risk factors for cardiovascular disease in humans. J Agric Food Chem 54:7364–7369

48. Ma G, Yang C, Qu Y, Wei H, Zhang T, Zhang N (2007) The flavonoid component isorhamnetin in vitro inhibits proliferation and induces apoptosis in Eca-109 cells. Chem-Biol Interact 167:153–160

49. Miyake Y, Shimoi K, Kumazawa S, Yamamoto K, Kinae N, Osawa T (2000) Identification and antioxidant activity of flavonoid metabolites in plasma and urine of eriocitrin-treated rats. J Agric Food Chem 48:3217–3224

50. Delporte C, Backhouse N, Erazo S, Negrete R, Vidal P, Silva X, López-Pérez JL, San Feliciano A, Muñoz O (2005) Anagesic-antiinflammatory properties of *Proustia pyrifolia*. J Ethnophamacol 99:119–124

51. Ley JP, Krammer G, Reinders G, Gatfield IL, Bertram HJ (2005) Evaluation of bitter masking flavanones from Herba Santa (Eriodictyon californicum (H. and A.) Torr., Hydrophyllaceae). J Agric Food Chem 53:6061–6066

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