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Functional identification of three regiospecific flavonoid O-methyltransferases in *Rhododendron delavayi* and their applications in the biotechnological production of methoxyflavonoids

Kyungha Lee¹, Seong Hee Bhoo², Sang-Won Lee^{2*} and Man-Ho Cho^{2*} 

Abstract

Rhododendrons produce a variety of methoxyflavonoids, including rarely found 3-methoxyflavonoids and 5-methoxyflavonoids. It was thus suggested that they have a series of regiospecific flavonoid O-methyltransferases (FOMTs). The 18 Class II O-methyltransferase (OMT) genes were retrieved from the *Rhododendron delavayi* genome, designating them as RdOMTs. A comprehensive biochemical characterization of RdOMTs was performed to identify functional FOMTs. The FOMT activity of recombinant RdOMTs was assayed with flavonoid substrates of different subclasses. Among the examined RdOMTs, RdOMT3, RdOMT10, and RdOMT12 showed FOMT activity for diverse flavonoids. In particular, RdOMT3 consumed only flavonols as a substrate. Structural analyses of the methylated products demonstrated that RdOMT3, RdOMT10, and RdOMT12 catalyze regiospecific methylation of flavonoids at the 3'/5', 3-, and 4'-hydroxyl groups, respectively. Their broad substrate spectrum and different regiospecificity suggest that these RdOMTs contribute to the formation of complex methoxyflavonoids in *R. delavayi*. Bioconversion of flavonoids using *E. coli* harboring each RdOMT demonstrated that RdOMT3, RdOMT10, and RdOMT12 are useful tools for the biotechnological production of valuable methoxyflavonoids, including the rarely found 3-methoxyflavonoids.

Keywords Flavonoid O-methyltransferase, RdOMT, Regiospecificity, Rhododendron, Bioconversion

Introduction

Rhododendron is a large genus of flowering plants in the family Ericaceae and widely distributed in the Northern Hemisphere [1–4]. In Asian, North American, and European traditional medicine, some *Rhododendron*

species have been used to treat various diseases, such as inflammation, pain, gastrointestinal disorders, colds, and asthma [1, 2, 5, 6]. The bioactive constituents of rhododendrons have been extensively isolated and identified. They are mainly flavonoids and diterpenoids [1, 2, 5, 6]. In addition to common flavonoids, such as kaempferol, quercetin, and myricetin, a variety of O-methylflavonoids and C-methylflavonoids were identified from rhododendron plants [1, 2, 5–7]. The C-methyl derivatives of naringenin and apigenin, such as farrerol (6,8-C-dimethylnaringenin), syzalterin (6,8-C-dimethylapigenin), and matteucinol (4'-O-methylfarrerol), were isolated in some rhododendron species, including *R. dauricum*, *R. concinnum*, and *R. fortunei* [5, 8–10].

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Common *O*-methylations of flavonoids occur at their 7-, 3'-, and 4'-hydroxy groups [11, 12]. The 5- and 3-*O*-methylations of flavonoids rarely occur in higher plants [11–13]. Interestingly, 5- and/or 3-methoxyflavonoids are often found in rhododendrons. Azaleatin (5-*O*-methylquercetin) was first isolated from a white azalea (*R. mucronatum* G. Don) [14]. Since then, other 5- and 3-*O*-methylflavonoids, such as 5-*O*-methylkaempferol, 5-*O*-methylmyricetin, 3-*O*-methylquercetin, and caryatin (3,5-*O*-dimethylquercetin), have been identified in many *Rhododendron* species, including *R. delavayi*, *R. austrinum*, *R. yedoense*, *R. ellipticum*, and *R. seniavinii* [1, 7, 13, 15–18].

In plants, the methylation of secondary metabolites is mainly accomplished by *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases [19, 20]. They can be involved in the *O*-, *C*-, and *N*-methylations of plant secondary metabolites. Although there are a few exceptions, flavonoid *O*-methyltransferase (FOMT) catalyzes the *O*-methylation of flavonoids in a regiospecific manner [11, 12, 19–21]. The complexity of methylation patterns in the rhododendron flavonoids implies that rhododendrons have an array of FOMTs with different regiospecificities. In this regard, *R. delavayi* OMT (RdOMT) genes were retrieved from the Rhododendron Plant Genome Database (RPGD) [4]. Biochemical studies of RdOMTs led us to identify three regiospecific FOMTs: RdOMT3, RdOMT10, and RdOMT12. The application of the regiospecific RdOMTs in the biotechnological production of methoxyflavonoids was also evaluated.

Materials and methods

In silico analysis of class II OMTs

The OMT genes in the *R. delavayi* genome were retrieved from the RPGD (<http://bioinfor.kib.ac.cn/RPGD/>) using the InterPro ID search tool, designating them as RdOMTs. The amino acid sequences of RdOMTs and other OMTs were aligned with Clustal-W to identify the signature motifs conserved in Class II OMTs [22]. The phylogenetic relationships among RdOMTs and other OMTs were inferred by the Neighbor-joining method using MEGA X [23–25].

Preparation of recombinant RdOMTs

The codons of the RdOMT genes were optimized for heterologous expression in *Escherichia coli* (Table S1). The synthetic RdOMT genes individually cloned in the pET-28b vector were obtained from Bionics (Seoul, Korea). Each RdOMT/pET-28a construct was transformed into *E. coli* BL21 (DE3) cells. The resulting *E. coli* transformants were grown in Luria–Bertani (LB) medium supplemented with kanamycin (25 µg/mL) at 37 °C. When the cell population reached an OD₆₀₀ of ~0.7, RdOMT

expression was induced with either 0.1 mM or 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at different temperatures ranging from 14 to 25 °C overnight. Recombinant RdOMTs were individually purified from the induction cultures by the method described by Park et al., 2024 [26].

Biochemical analysis

The FOMT assay of recombinant RdOMTs and a high-performance liquid chromatography (HPLC) analysis were performed by the method described by Park et al., 2024 [26]. RdOMT3 reactions for kinetic analysis were carried out with eriodictyol, luteolin, quercetin, and rhamnetin. Concentrations of eriodictyol, luteolin, and rhamnetin for the RdOMT3 kinetics ranged from 0.5 µM to 50 µM. The quercetin concentrations were 1–50 µM. Kaempferol, quercetin, and isorhamnetin were used for the kinetic analysis of RdOMT10. Substrate concentrations for the RdOMT10 reactions were 0.7–10 µM. The kinetic analysis of RdOMT12 was performed with naringenin, apigenin, luteolin, and kaempferol. The concentrations of the substrates for the kinetic analysis of RdOMT12 ranged from 0.5 µM to 50 µM.

Structural analysis of the methylated products

Liquid chromatography-mass spectrometry (LC–MS) analysis of authentic 3'- and 4'-methoxyflavonoids and the reaction mixtures of RdOMT3 and RdOMT12 were carried out by the method described by Park et al., 2024 [26]. The methylated products of the RdOMT10 reactions were isolated by HPLC equipped with a preparative SunFire C18 column (10 mm × 150 mm, Waters, Milford, MA, USA) using a linear gradient of 25–60% acetonitrile in 3% acetic acid–water for 25 min at a flow rate of 3 mL/min with detection at 280 nm. The ¹H, ¹³C, and heteronuclear multiple bond correlation (HMBC) NMR spectra of the isolated products were recorded with an Avance III HD spectrometer (Bruker, Billerica, MA, USA) operating at 600 MHz (for ¹H) and 150 MHz (for ¹³C) in acetone-*d*₆.

3-*O*-Methylkaempferol. ¹H NMR δ (ppm) 6.25 (d, *J*=1.9 Hz, H-6), 6.50 (d, *J*=1.9 Hz, H-8), 8.03 (d, *J*=8.9 Hz, H-2', 6'), 7.02 (d, *J*=8.9 Hz, H-3', 5'), 3.87 (s, 3-OCH₃); ¹³C NMR δ 156.92 (C-2), 139.25 (C-3), 179.60 (C-4), 163.31 (C-5), 99.47 (C-6), 164.98 (C-7), 94.56 (C-8), 157.94 (C-9), 105.98 (C-10), 122.75 (C-1'), 131.28 (C-2'), 116.48 (C-3'), 160.99 (C-4'), 116.48 (C-5'), 131.28 (C-6'), 60.28 (3-OCH₃).

3-*O*-Methylquercetin. ¹H NMR δ 6.25 (d, *J*=1.9 Hz, H-6), 6.49 (d, *J*=1.9 Hz, H-8), 7.70 (d, *J*=2.1 Hz, H-2'), 6.99 (d, *J*=8.5 Hz, H-5'), 7.58 (dd, *J*=8.5, 2.1 Hz, H-6'), 3.86 (s, 3-OCH₃); ¹³C NMR δ 156.82 (C-2), 139.36 (C-3), 179.60 (C-4), 163.29 (C-5), 99.44 (C-6), 164.94 (C-7), 94.49 (C-8), 157.89 (C-9), 105.97 (C-10), 123.11 (C-1'),

116.35 (C-2'), 145.94 (C-3'), 149.18 (C-4'), 116.42 (C-5'), 122.21 (C-6'), 60.24 (3-OCH₃).

3-O-Methylisorhamnetin. ¹H NMR δ 6.25 (d, *J*=1.9 Hz, H-6), 6.51 (d, *J*=1.9 Hz, H-8), 7.77 (d, *J*=2.0 Hz, H-2'), 7.00 (d, *J*=8.5 Hz, H-5'), 7.68 (dd, *J*=8.5, 2.0 Hz, H-6'), 3.89 (s, 3-OCH₃), 3.95 (s, 3'-OCH₃); ¹³C NMR δ 156.71 (C-2), 139.35 (C-3), 179.56 (C-4), 163.26 (C-5), 99.52 (C-6), 165.20 (C-7), 94.67 (C-8), 157.92 (C-9), 105.88 (C-10), 122.92 (C-1'), 112.71 (C-2'), 148.34 (C-3'), 150.51 (C-4'), 116.15 (C-5'), 123.41 (C-6'), 60.31 (3-OCH₃), 56.48 (3'-OCH₃).

Regiospecific methylation of flavonoids using whole-*E. coli* cells

Expression of each RdOMT in *E. coli* cells was induced with 0.5 mM IPTG at 25 °C for 4 h. The cells were harvested and resuspended in the same volume of fresh LB medium. The bioconversion by RdOMT3 was carried out with eriodictyol, luteolin, and quercetin. Kaempferol, quercetin, and isorhamnetin were used for the RdOMT10 reactions. The substrates used in the bioconversion by RdOMT12 were naringenin, apigenin, and luteolin. Flavonoid substrates (50 μM) were individually added to the fresh cell suspension and further incubated at 25 °C. An aliquot of the culture was harvested at the selected time points and centrifuged to obtain a cell-free medium. The medium was extracted with ethyl acetate and analyzed by the method described by Park et al., 2024 [26].

Results and discussion

Class II OMT family in the *R. delavayi* genome

OMTs are a large group of enzymes that belong to the SAM-dependent methyltransferase superfamily. They are involved in the methylation of diverse secondary metabolites, including flavonoids, phenylpropanoids, stilbenes, and alkaloids [19, 20]. Two classes of SAM-dependent OMT have been known to catalyze the *O*-methylation of phenolic compounds. The cation-dependent Class I OMT participates in the methylation of hydroxycinnamoyl-CoAs during monolignol biosynthesis [20]. Class II OMTs catalyze the methylation of a variety of phenolic compounds, including caffeic acids, flavonoids, and isoflavonoids [19–21]. To identify biochemically active FOMTs, OMT genes were retrieved from the *R. delavayi* genome in the RPGD. It has been well known that both an *O*-methyltransferase domain (InterPro ID: IPR001077) and a plant methyltransferase dimerisation domain (InterPro ID: IPR012967) are conserved in Class II OMT that is involved in the *O*-methylation of flavonoids and isoflavonoids [19–21, 27–29]. A search for the InterPro ID IPR001077 in the RPGD demonstrated that there are 18 tentative Class II OMTs (RdOMT1–RdOMT18) in the *R. delavayi* genome (Fig. 1). Most RdOMTs also

have the dimerisation domain, except for RdOMT16 and RdOMT17 (Fig. 1). The gene lengths of the functionally identified Class II OMTs are about 1100 nucleotides long, encoding proteins of approximately 40 kDa molecular mass [12, 19–21]. The open reading frames of RdOMT15, RdOMT16, RdOMT17, and RdOMT18 are significantly shorter than typical Class II OMTs and the other RdOMTs (Table S1). RdOMT15, RdOMT17, and RdOMT18 have abnormally short *O*-methyltransferase domains compared to the other RdOMTs (Fig. 1). The defects in the key domains in RdOMT15–RdOMT18 suggest that they are unlikely to encode normal Class II OMTs. Therefore, the biochemical functions of 14 RdOMTs (RdOMT1–RdOMT14) were examined in the present study.

In our previous study, a phylogenetic analysis categorized FOMTs into two groups, Group I and II [26]. Another small group of FOMTs was found in the present study and named Group III (Fig. S1). Group I includes seven RdOMTs (RdOMT1–RdOMT5, RdOMT9, and RdOMT14), caffeic acid OMTs (COMTs), and a majority of functionally identified FOMTs (Fig. S1). Some COMTs have been reported to have FOMT activity. Recombinant *Arabidopsis thaliana* COMT (AtCOMT) was shown to catalyze the 3'-*O*-methylation of flavonols [30]. Rice COMT (OsCOMT) was reported to have flavonoid 3'-OMT (F3'OMT) activity [31]. Most Group I FOMTs are flavonoid 3'- and/or 5'-OMTs, and a few of them are flavonoid 7-OMTs (Fig. S1) [26, 30–35]. RdOMT3 is closely related to COMTs and Group I FOMTs. Separated from Group I, RdOMT10 is categorized with alfalfa ChOMT and *Solanum habrochaites* MOMTs (ShMOMT1 and ShMOMT3), designating Group III (Fig. S1). Isoflavonoid OMTs (IFOMTs) and some FOMTs were early separated from Group I and III FOMTs. These FOMTs were designated as Group II (Fig. S1). The Group II members are flavonoid 4'- and/or 7-OMTs [36–39]. RdOMT6, RdOMT7, RdOMT8, RdOMT11, RdOMT12, and RdOMT13 were included in Group II (Fig. S1).

RdOMT3, RdOMT10, and RdOMT12 are regiospecific FOMTs Three biochemically active RdOMTs among the class II OMTs of *R. delavayi*

It has been reported that only a few OMT genes encode a biochemically active OMT, although many OMTs exist in plant genomes [26, 32, 40, 41]. RdOMTs were heterologously expressed in *E. coli*, and their FOMT activity toward flavonoids was examined to identify functional OMTs. Of the examined RdOMTs, RdOMT2, RdOMT3, RdOMT4, RdOMT5, RdOMT6, RdOMT8, RdOMT9, RdOMT10, and RdOMT12 were expressed as a soluble form in *E. coli* BL21 cells at 18 or 25 °C of growth temperature by 0.5 mM IPTG

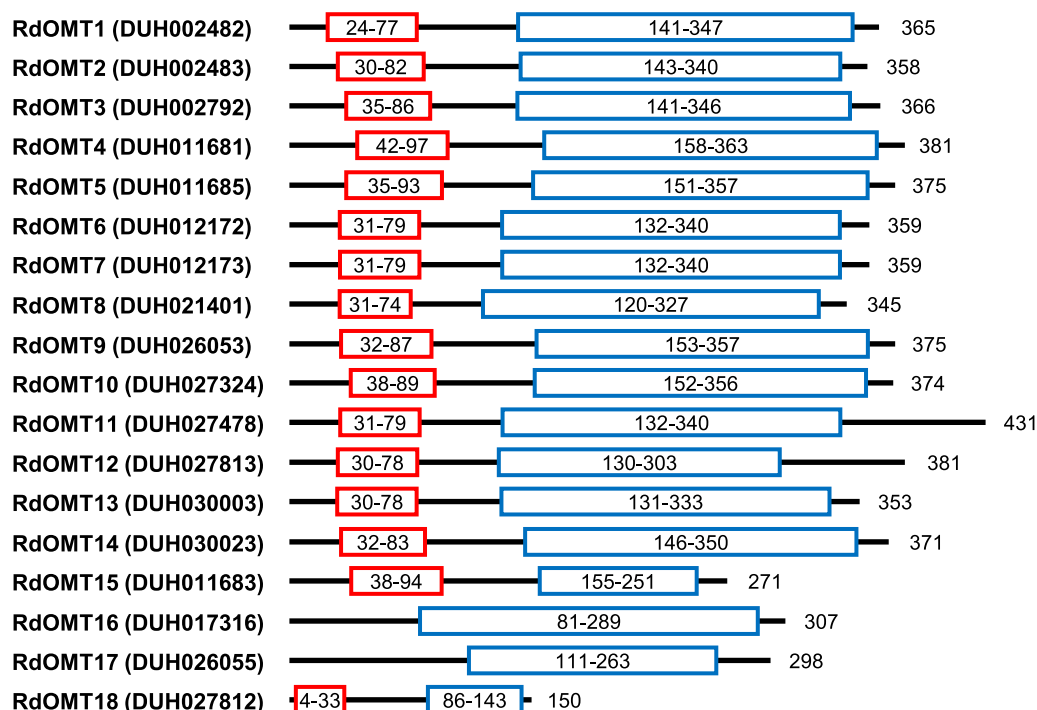


Fig. 1 Conserved protein domains in the Class II OMTs of *R. delavayi*. Gene IDs of Class II OMTs in the *R. delavayi* genome retrieved from the RPGD are in parentheses. The plant methyltransferase dimerisation domains and O-methyltransferase domains are indicated in red and blue boxes, respectively. The amino acid positions of the domains are included in the boxes. The conserved domain was searched in InterPro (<https://www.ebi.ac.uk/interpro/>). The polypeptide lengths of RdOMTs are on the right

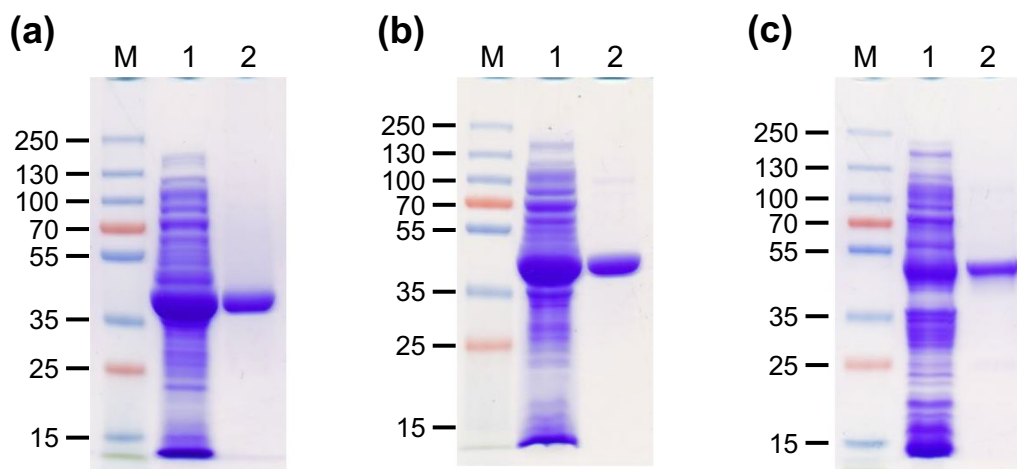


Fig. 2 Purification of the recombinant RdOMT3 (a), RdOMT10 (b), and RdOMT12 (c). M, size marker; 1, total protein extract from the induction cultures; 2, purified recombinant RdOMT protein

(Fig. 2 and Fig. S2). The recombinant RdOMTs were successfully purified by Ni^{2+} -affinity chromatography (Fig. 2 and Fig. S2). RdOMT1, RdOMT7, RdOMT11, RdOMT13, and RdOMT14 were expressed in only insoluble form in *E. coli* BL21 cells at all attempted induction conditions.

FOMT activity of the purified recombinant RdOMTs was assayed with diverse flavonoids of different subclasses. The HPLC analysis of the RdOMT reaction mixtures demonstrated that RdOMT3, RdOMT10, and RdOMT12 used flavonoids and produced the respective methylated flavonoids (Figs. 3 and 4). This result

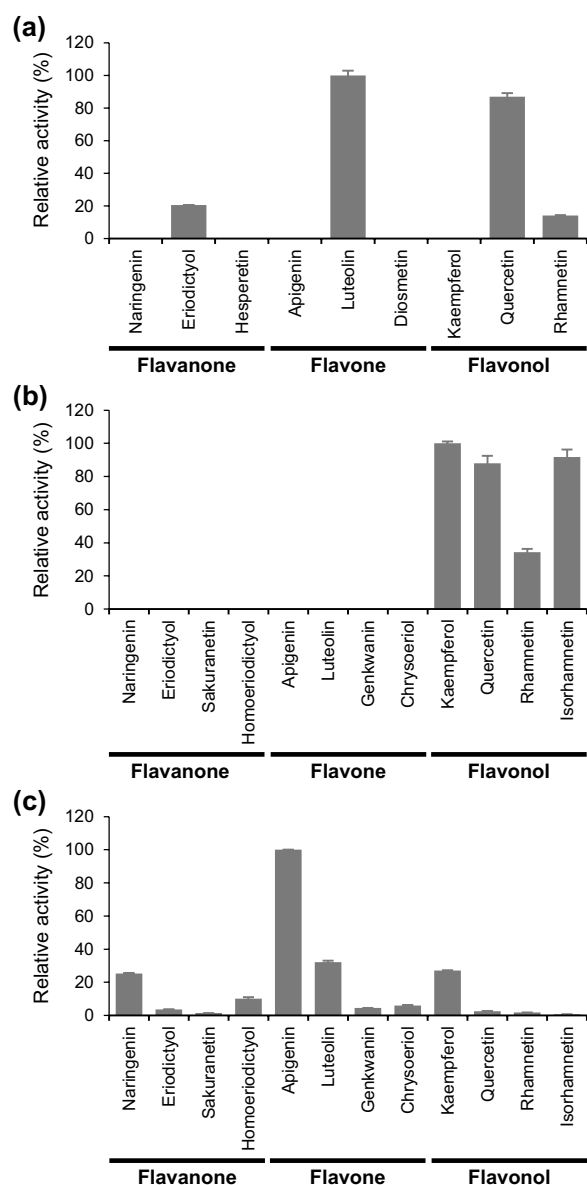


Fig. 3 FOMT activities of recombinant RdOMT3 (a), RdOMT10 (b), and RdOMT12 (c) for flavonoid substrates. Relative activity indicates the ratio of FOMT activity of RdOMTs for flavonoid substrates relative to that for the selected substrates. The FOMT activity of RdOMT3, RdOMT10, and RdOMT12 for luteolin, kaempferol, and apigenin was set at 100%, respectively. The FOMT activities of RdOMTs for flavonoids were measured at substrate concentrations of 50 μ M. Duplicated experiments were performed. The results represent the mean relative activities and standard deviations

exhibits that RdOMT3, RdOMT10, and RdOMT12 are biochemically active FOMTs among the Class II OMTs of *R. delavayi*. No products were detected in the reactions of RdOMT2, RdOMT4, RdOMT5, RdOMT6, RdOMT8, and RdOMT9 to all flavonoids examined.

Structural studies of alfalfa COMT (MsCOMT) and ChOMT demonstrated that three residues (His, Glu/Asp, and Glu) participate in the catalysis of Class II OMT [28, 29]. The catalytic residues are conserved in RdOMT3 (His270, Glu298, and Glu330), RdOMT10 (His281, Glu309, and Glu340), and RdOMT12 (His263, Asp293, and Glu347) (Fig. S3). The SAM-binding motifs (SAM-A, B, and C) and COMT motifs (COMT-I, J, K, and L) were suggested to be conserved in the Class II OMT [21]. An alignment of the amino acid sequences of RdOMTs and other functionally identified FOMTs showed that RdOMT3, RdOMT10, and RdOMT12 have all the signature motifs (Fig. S3). The SAM binding motifs were found to be highly conserved in functional FOMTs, including RdOMT3, RdOMT10, and RdOMT12. Minor substitutions were observed in the SAM-B and C motifs of the Group II and III FOMTs (Fig. S3). A few insertions were found in the SAM-C motif of the Group II FOMT RdOMT12 and CrOMT6 (Fig. S3). This finding agrees with the dependence of FOMTs on SAM. The COMT motifs are well conserved in RdOMT3, AtCOMT, and CaFOMT (Fig. S3). It is consistent with the close relationship between COMTs and Group I FOMTs. In RdOMT10, ShMOMT3, RdOMT12, and CrOMT6, some substitutions were found in the COMT motifs (Fig. S3).

RdOMT3 is a flavonoid 3'/5'-OMT

The FOMT reactions have been known to be mainly regiospecific [11, 12, 32–34]. In the phylogenetic tree, RdOMT3 was included in Group I and closely related to flavonoid 3'/5'-OMTs (F3'/5'-OMTs) (Fig. S1). Although RdOMT3 methylated diverse flavonoids examined, it did not use naringenin, apigenin, or kaempferol (Fig. 3a). Compared to other flavonoids, naringenin, apigenin, and kaempferol have no 3'-OH group. It thus implied that RdOMT3 requires the 3'-OH on the flavonoid backbones for methylation. As expected, the methylated products of eriodictyol, luteolin, and quercetin by RdOMT3 exhibited an identical retention time to the authentic 3'-methoxyflavonoids, homoeriodictyol (3'-O-methyleriodictyol), chrysoeriol (3'-O-methyllyuteolin), and isorhamnetin (3'-O-methylquercetin), respectively (Fig. 4a–c). The 3'-O-methylation of flavonoids by RdOMT3 was further confirmed by a comparison of the mass spectra for the reaction products and the authentic 3'-methoxyflavonoids. In the LC–MS analysis of the RdOMT3 reaction mixtures, the methylated products and the authentic 3'-methoxyflavonoids exhibited identical molecular masses and fragmentation patterns (Fig. S4). Because the 3'-OH and 5'-OH on flavonoids are chemically equivalent, most F3'OMTs can catalyze the 5'-O-methylation [27, 30]. In this regard, the FOMT activity of RdOMT3 for laricitrin (3'-O-methylmyricetin) was

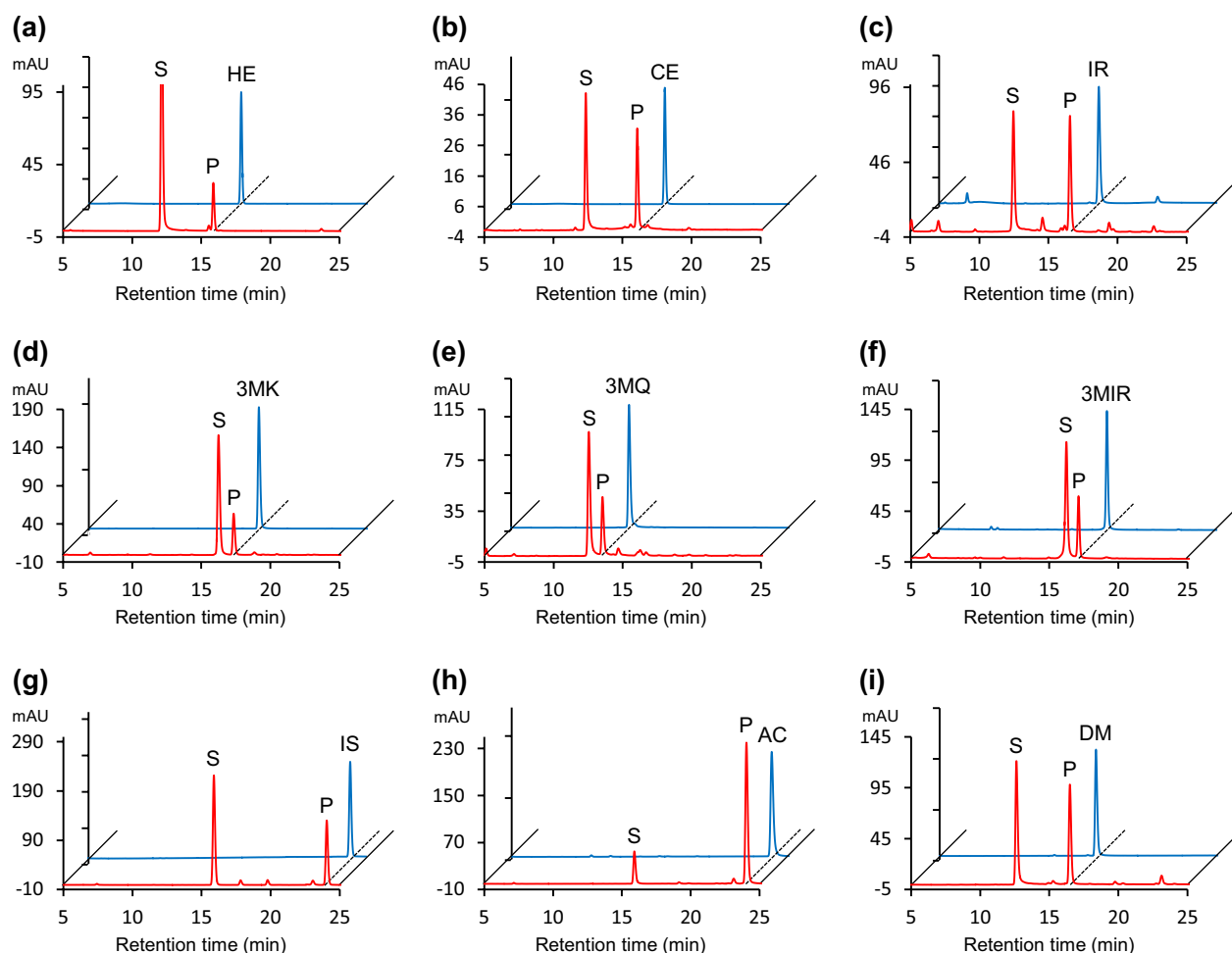


Fig. 4 Representative HPLC chromatograms of the RdOMT reactions. (a–c) The RdOMT3 reactions with eriodictyol (a), luteolin (b), and quercetin (c). (d–f) FOMT activity of RdOMT10 for kaempferol (d), quercetin (e), and isorhamnetin (f). (g–i) The RdOMT12 reactions with naringenin (g), apigenin (h), and luteolin (i). The HPLC chromatograms of reaction mixtures and authentic methoxyflavonoids are in red and blue lines, respectively. An HPLC chromatogram of the 3'-O-methylflavonoid homoeriodictyol (HE), chrysoeriol (CE), and isorhamnetin (IR), the 3-O-methylflavonoid 3-O-methylkaempferol (3MK), 3-O-methylquercetin (3MQ), and 3-O-methylisorhamnetin (3MIR), and the 4'-O-methylflavonoid isosakuranetin (IS), acacetin (AC), and diosmetin (DM), was individually inserted. S, substrate; P, product

examined. An HPLC analysis exhibited that RdOMT3 used laricitrin and produced syringetin (3',5'-O-dimethylmyricetin) (Fig. S5). These findings led us to conclude that RdOMT3 is an F3'/5'OMT.

RdOMT10 catalyzes the 3-O-methylation of flavonoids

RdOMT10 is categorized into Group III together with ShMOMT1, ShMOMT3, and ChOMT (Fig. S1). The FOMTs in Group III exhibited diverse regiospecificities. ShMOMT1 and ShMOMT3 were reported to be an F3'/5'OMT and a flavonoid 3-OMT (F3OMT), respectively [38, 42]. ChOMT catalyzes the 2'-O-methylation of chalcone [43]. Of different subclasses of flavonoids, RdOMT10 consumed only flavonols as a substrate and yielded methylated products (Figs. 3b and 4d–f).

The restricted consumption of flavonols suggests that RdOMT10 likely catalyzes the 3-O-methylation of flavonoids. Because of the requirement for the 3-OH group, F3OMT is rarely found in plants [12]. A few F3OMTs have been identified in plants. *C. roseus* OMT1 (CrOMT1) was reported to methylate phenylpropanoids, such as caffeate and 5-hydroxyferulate, and also catalyze the methylation of flavonols to a lesser extent [44]. The strict dependence on flavonols as a substrate suggests that CrOMT1 is likely a F3OMT. An OMT was partially purified from *Serratula tinctoria* leaves and characterized as a flavonol 3-OMT [45]. ShMOMT3 and SiMOMT3 from wild and cultivated tomatoes were found to catalyze the 3-O-methylation of flavonols and 7/3'/4'/5'-O-methylflavonols [42].

Because no authentic 3-methoxyflavonoids were available, the methylated products of kaempferol, quercetin, and isorhamnetin were isolated from the RdOMT10 reactions by a preparative HPLC to confirm the regio-specificity. The NMR analyses of the isolated methylflavonoids were performed to determine the position of methylation. The methoxy proton signals of the methylated products, methylkaempferol and methylquercetin, were observed at δ 3.87 and δ 3.86, respectively, in the ^1H NMR spectra (see Method). The ^1H NMR spectrum of the methylisorhamnetin showed two methoxy proton signals. One methoxy proton signal of 3'-OCH₃ appeared at δ 3.95, and another signal was observed at δ 3.89. The methoxy signal at δ 3.95 was found to correlate with the C-3' signal at δ 148.34 in the HMBC spectrum (Fig. S6). The C-3 signals of the methylated products, methylkaempferol, methylquercetin, and methylisorhamnetin, were observed at δ 139.25, δ 139.36, and δ 139.35, respectively. As expected, the proton signals of the newly added methyl group were observed to correlate with the C-3 signals of the methylated products in the HMBC spectra (Fig. S6). This result demonstrated that the methylation products of kaempferol, quercetin, and isorhamnetin by RdOMT10 are isokaempferide (3-O-methylkaempferol), 3-O-methylquercetin, and 3-O-methylisorhamnetin (3, 3'-O-dimethylquercetin), respectively. The strict dependence on flavonol substrates and the HMBC correlations between the methoxy signals and the C-3 signals indicate that RdOMT10 catalyzes the 3-O-methylation of flavonols.

RdOMT12 is a flavonoid 4'-OMT

Known FOMTs that belonged to Group II were shown to have flavonoid 4'- and/or 7-OMT activities (Fig. S1) [36–39]. RdOMT12 is a member of Group II. The FOMT activity assay showed that RdOMT12 catalyzes the methylation of all flavonoid substrates examined (Fig. 3c). The 4'-, 5-, and 7-OH groups are common in flavonoids because they are synthesized by the condensation of *p*-coumaroyl-CoA and malonyl-CoAs, followed by an isomerization (Fig. 5) [46–49]. Therefore, these OH-groups were suggested to be the possible positions for the RdOMT12 methylation. The retention times of the methylated products from naringenin, apigenin, and luteolin by RdOMT12 were the same as those of the authentic 4'-methoxyflavonoids, isosakuranetin (4'-O-methylnaringenin), acacetin (4'-O-methylapigenin), and diosmetin (4'-O-methyllyluteolin), in the HPLC analysis, respectively (Fig. 4g-i). This result implies that RdOMT12 catalyzes the 4'-O-methylation of flavonoids. An LC-MS analysis also showed that the molecular masses and fragmentation patterns of the methylated products from naringenin, apigenin, and luteolin by RdOMT12 were identical

with those of isosakuranetin, acacetin, and diosmetin, respectively (Fig. S7). It was therefore concluded that RdOMT12 is a flavonoid 4'-OMT (F4'OMT).

Substrate preference of RdOMT3, RdOMT10, and RdOMT12

It has been well established that FOMTs have a broad substrate spectrum for flavonoids [11, 12, 20]. In the present study, a variety of flavonoids and methoxyflavonoids were used in the FOMT assay of RdOMTs. Of the flavonoid substrates examined, RdOMT3 showed strong FOMT activity for luteolin and quercetin at concentrations of 50 μM (Fig. 3a). In the same condition, the activity of RdOMT3 for eriodictyol was about five-fold lower than that for luteolin. Of the mono-methoxyflavonoid substrates, RdOMT3 consumed rhamnetin and laricitrin (Fig. 3a). However, no methylated products from hesperetin (4'-O-methyleriodictyol) and diosmetin (4'-O-methyllyluteolin) were detected in the RdOMT3 reactions, suggesting that the vicinal methyl group likely interferes with the 3'-O-methylation by RdOMT3. A kinetic analysis was carried out to further evaluate the efficiency of the RdOMT3 reaction for the selected substrates (eriodictyol, luteolin, quercetin, and rhamnetin). The K_m values of RdOMT3 for eriodictyol, luteolin, and rhamnetin were 0.75 μM , 1.35 μM , and 0.87 μM , respectively (Table 1). This result suggests that RdOMT3 has a similar binding affinity to these substrates. The K_m value of RdOMT3 for quercetin was quite higher than that for the other substrates. RdOMT3 showed the highest $V_{\max}$ value for quercetin, followed by luteolin (Table 1). The $V_{\max}$ values of RdOMT3 for eriodictyol and rhamnetin were lower than those for quercetin and luteolin. In parallel with its relative activity, RdOMT3 showed the highest enzymatic efficiency to luteolin, with a k_{cat}/K_m value of $9.53 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ among the examined substrates (Table 1). These findings indicated that luteolin is a preferable substrate for RdOMT3, although it can use diverse flavonoids.

Because of the requirement for the 3-OH group, substrates for RdOMT10 were limited to flavonols. RdOMT10 was shown to methylate kaempferol and quercetin to similar extents in the saturated conditions (Fig. 3b). It also methylated the mono-methoxyflavonol rhamnetin and isorhamnetin. In the same condition, the FOMT activity of RdOMT10 for isorhamnetin was comparable to that for quercetin. The relative activity of RdOMT10 for rhamnetin was quite weaker than that for the other flavonols (Fig. 3b). In parallel with its relative activity, RdOMT10 has comparable $V_{\max}$ values for kaempferol, quercetin, and isorhamnetin (Table 1). The K_m values of RdOMT10 for quercetin and isorhamnetin were 1 μM and 1.06 μM , respectively, indicating that it has a similar binding affinity for these substrates

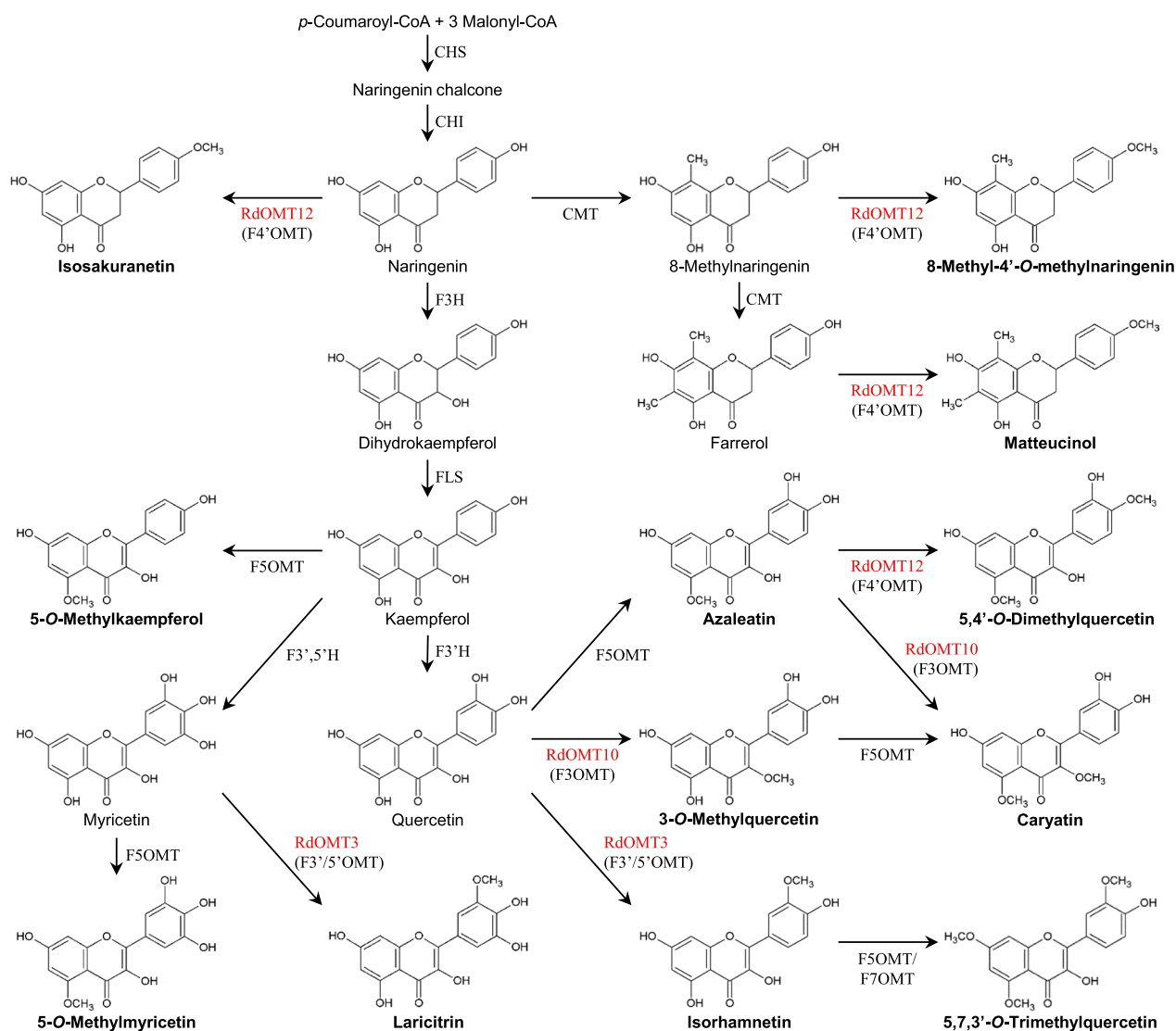


Fig. 5 Possible involvement of regiospecific FOMTs in the formation of complex methoxyflavonoids in rhododendrons. Three regiospecific RdOMTs are in red. Methoxyflavonoids identified in rhododendrons are in bold. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; F3'H, flavonoid 3'-hydroxylase; F5'H, flavonoid 5'-hydroxylase; F3'/5'H, flavonoid 3'/5'-hydroxylase; CMT, C-methyltransferase; F3OMT, flavonoid 3-O-methyltransferase; F3'/5'OMT, flavonoid 3'/5'-O-methyltransferase; F4'OMT, flavonoid 4'-O-methyltransferase; F5OMT, flavonoid 5-O-methyltransferase; F7OMT, flavonoid 7-O-methyltransferase

(Table 1). RdOMT10 showed a high K_m value for kaempferol compared to that for quercetin and isorhamnetin (Table 1). Therefore, k_{cat}/K_m values for quercetin and isorhamnetin were about tenfold higher than those for kaempferol (Table 1). Taken together, RdOMT10 prefers quercetin and isorhamnetin as substrates, followed by kaempferol.

Although RdOMT12 catalyzed the methylation of all examined flavonoids, it showed different FOMT activities for substrates. RdOMT12 exhibited the strongest activity for apigenin under the saturated condition (Fig. 3c). In

the same condition, RdOMT12 showed moderate FOMT activities for naringenin, luteolin, and kaempferol, with relative activities of 25.32, 32.25, and 27.06%, respectively, to the activity for apigenin (Fig. 3c). The activity of RdOMT12 on the other flavonoids was less than 10% of that of apigenin. In the kinetic study, RdOMT12 had the lowest K_m value of 0.3 μ M for apigenin (Table 1). The K_m values for naringenin, luteolin, and kaempferol were 3.11 μ M, 0.5 μ M, and 1.04 μ M, respectively. RdOMT12 exhibited comparable V_{max} values for apigenin, naringenin, luteolin, and kaempferol, ranging from

Table 1 Kinetic constants of the RdOMT3, RdOMT10, and RdOMT12 reactions^a

Enzyme	Substrate	K_M (μM)	$V_{\max}$ ($\text{pmol min}^{-1} \mu\text{g}^{-1}$)	k_{cat} (min^{-1})	k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$)
RdOMT3	Eriodictyol	0.75 ± 0.12	5.00 ± 0.24	0.211	4.71×10^3
	Luteolin	1.35 ± 0.07	18.3 ± 1.05	0.772	9.53×10^3
	Quercetin	6.23 ± 1.42	37.2 ± 4.46	1.566	4.19×10^3
	Rhamnetin	0.87 ± 0.06	4.76 ± 0.06	0.200	3.84×10^3
RdOMT10	Kaempferol	7.09 ± 1.63	19.3 ± 0.86	0.824	1.94×10^3
	Quercetin	1.00 ± 0.14	29.6 ± 0.81	1.262	2.10×10^4
	Isorhamnetin	1.06 ± 0.20	24.0 ± 2.03	1.024	1.62×10^4
RdOMT12	Naringenin	3.11 ± 0.20	3.01 ± 0.11	0.134	7.19×10^2
	Apigenin	0.30 ± 0.08	3.21 ± 0.14	0.144	7.94×10^3
	Luteolin	0.50 ± 0.06	1.76 ± 0.08	0.079	2.63×10^3
	Kaempferol	1.04 ± 0.07	1.07 ± 0.12	0.048	7.69×10^2

^a Three independent experiments were performed, and the results are presented

$1.07 \text{ pmol min}^{-1} \mu\text{g}^{-1}$ to $3.21 \text{ pmol min}^{-1} \mu\text{g}^{-1}$ (Table 1). The overall enzyme efficiency of RdOMT12 for apigenin was the highest among the examined flavonoids. The kinetic parameters and relative activity indicated that apigenin is the best substrate for RdOMT12.

Regiospecific FOMTs contribute to the formation of complex methoxyflavonoids in rhododendrons

Extensive phytochemical analyses of rhododendrons have shown that they produce a wide array of flavonoids, from the common flavonoid naringenin, kaempferol, and quercetin to C- and O-methylflavonoids [1, 5–7, 50]. The flavanone naringenin is an entry point of the flavonoid metabolism and is synthesized from a *p*-coumaroyl-CoA and three malonyl-CoAs by consecutive actions of chalcone synthase and chalcone isomerase (Fig. 5) [46–49]. Therefore, flavonoids generally contain the 5-, 7-, and 4'-OH groups. In addition, flavonoid backbones are often hydroxylated at the 3'-position [46, 47]. These OH-groups, except for 5-OH, are common sites of the flavonoid O-methylations [12, 13]. The 3'-methoxyflavonoid isorhamnetin and laricitrin and the 4'-methoxyflavonoid isosakuranetin and hesperetin were identified in some *Rhododendron* species [1, 8, 51–56]. In addition to these common O-methylations, 3- and 5-O-methylations have often been found in the rhododendron flavonoids. 3-O-Methylquercetin was isolated from *R. delavayi* and *R. luteum* [17, 57]. Azaleatin, 5-O-methylkaempferol, and 5-O-methylmyricetin are widely found in rhododendrons [5, 7, 13]. Besides mono-methoxyflavonoids, di-methoxyflavonoids (caryatin, 5,4'-O-dimethylquercetin, and 7,4'-O-dimethylfarrerol) and 5,7,3'-O-trimethylquercetin have been identified in *Rhododendron* species, including *R. delavayi*, *R. austrinum*, *R. ellipticum*, *R. seniavinii*, and *R. hainanense* [7, 8, 13, 15, 17, 18].

The complexity of methoxyflavonoids suggests that rhododendrons need to have a series of FOMTs because of their regiospecific nature (Fig. 5). In the present study, we comprehensively investigated the Class II OMTs in the *R. delavayi* genome and identified three biochemically functional RdOMTs. The functional RdOMTs include a rarely found F3OMT, RdOMT10, as well as the F3'/5'OMT RdOMT3 and the F4'OMT RdOMT12. Phytochemical studies have been reported to identify diverse methoxyflavonoids, such as 3-O-methylquercetin, 3,5-O-dimethylquercetin, and isorhamnetin, from *R. delavayi* [17, 53]. Its 3-O-methylation activity and broad substrate spectrum suggest that RdOMT10 likely participates in the formation of 3-methoxyflavonoids in *R. delavayi*. RdOMT3 and RdOMT12 were also likely involved in the 3', 4', and/or 5'-O-methylation of flavonoids, leading to the synthesis of common methoxyflavonoids, such as isorhamnetin, in *R. delavayi*. The possible roles of regiospecific FOMTs in the formation of complex methoxyflavonoids in rhododendrons are summarized in Fig. 5.

Applications of regiospecific RdOMTs in the biotechnological production of methoxyflavonoids

Bioconversion using whole-cell microorganisms bearing biosynthetic enzymes has been considered a promising method for the production of valuable natural products [11, 58]. *E. coli* cells harboring *Plagiochasma appendiculatum* F4'OMT (PaF4'OMT) were reported to be used in the regiospecific conversion of apigenin to acacetin [59]. The flavonoid 7-O-methyltransferase PaOMT-7 was applied in the production of the 7-methoxyflavonoids from luteolin, kaempferol, apigenin, and quercetin [60].

Although a few F3OMTs were identified, their applications in the biotechnological production of 3-methoxyflavonoids have not been reported yet. In this regard, the

bioconversion of kaempferol, quercetin, and isorhamnetin using *E. coli* cells bearing RdOMT10 was attempted to produce the respective 3-methoxyflavonoids (Fig. 6d–f). The HPLC analysis of the reaction mixtures showed that the examined substrates were almost consumed within 2 h. RdOMT10-transformed *E. coli* cells converted isorhamnetin to an equivalent amount of 3-*O*-methylisorhamnetin after 4 h of bioconversion (Fig. 6f). The production of isokaempferide and 3-*O*-methylquercetin from kaempferol and quercetin reached the highest levels at 3 h and 2 h, with maximum yields of 84.5% and 64.3%, respectively (Fig. 6d, e). This result implies that the *E. coli* cells bearing RdOMT10 are a useful whole-cell system for the production of 3-methoxyflavonoids.

Production of 3'-methoxyflavonoids and 4'-methoxyflavonoids was tried with RdOMT3 and RdOMT12, respectively. Eriodictyol, luteolin, and quercetin were individually added to the culture of *E. coli* cells harboring RdOMT3 to evaluate the 3'-methoxyflavonoid production. The RdOMT3-transformed cells rapidly consumed luteolin and quercetin within 2 h (Fig. 6b, c). Eriodictyol was gradually used by the *E. coli* cells through the bio-transformation period (Fig. 6a). This result agrees well with the relative activity of RdOMT3 on the examined substrates. The amount of chrysoeriol and isorhamnetin reached maximum levels at 2 h of bioconversion and then decreased (Fig. 6b, c). The maximum yields of chrysoeriol and isorhamnetin from luteolin and quercetin were

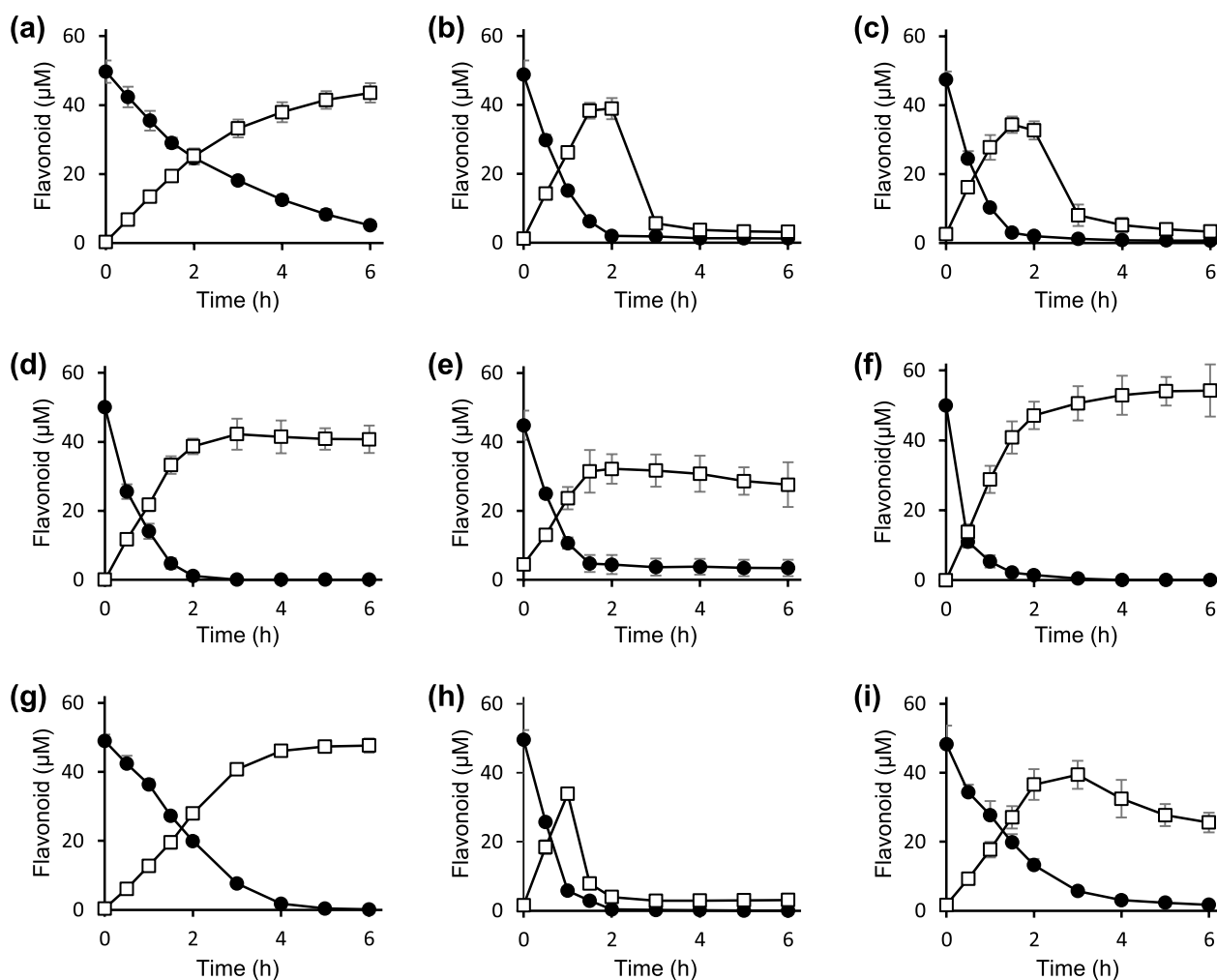


Fig. 6 Regiospecific production of methoxyflavonoids using whole *E. coli* cells expressing RdOMT. (a–c) The RdOMT3-transformed *E. coli* cells were used in the production of the 3'-methoxyflavonoids from eriodictyol (a), luteolin (b), and quercetin (c). (d–f) The 3-methoxyflavonoids were produced from kaempferol (d), quercetin (e), and isorhamnetin (f) by *E. coli* cells harboring RdOMT10. (g–i) The production of the 4'-methoxyflavonoids from naringenin (g), apigenin (h), and luteolin (i) was carried out with the RdOMT12-transformed *E. coli* cells. Three independent experiments were performed, and the results were presented

77.9% and 68.7%, respectively. The yield of homoeriodictyol from eriodictyol was 87.1% at 6 h of bioconversion.

E. coli cells harboring RdOMT12 were used in the production of the 4'-methoxyflavonoids from naringenin, apigenin, and luteolin. Naringenin was successfully converted to isosakuranetin by RdOMT12-transformed *E. coli* cells without significant loss (Fig. 6g). RdOMT12-transformed *E. coli* cells showed the highest initial bioconversion rate to apigenin among the examined substrates (Fig. 6g–i). The level of acacetin from apigenin reached its maximum at 1 h of bioconversion and rapidly decreased (Fig. 6h). This result suggests that the bioconversion time needs to be carefully controlled for a better production yield of acacetin. The production of diosmetin from luteolin by RdOMT12-transformed *E. coli* cells reached its highest level at 3 h of biotransformation, with a yield of 78.8% (Fig. 6i).

The health-beneficial properties of methoxyflavonoids have been consistently reported [61–69]. 3-*O*-Methylquercetin was reported to have chemoprevention effects against skin and esophageal cancer by targeting the extracellular signal-related kinase and mitogen-activated protein kinase pathways [65, 66]. Several studies have demonstrated the anti-cancer and anti-inflammatory activities of isokaempferide [64, 69]. Methoxyflavonoids, such as 3-*O*-methylisorhamnetin, sakuranetin, ponciretin, and rhamnazin, have been reported to have antimicrobial activity against bacteria and/or fungi [61, 70–72]. 3'-Methoxyflavonoids and 4'-methoxyflavonoids are widely found in plants. They have been suggested as potent therapeutic agents to treat cancer, inflammation, infections, and other ailments [67, 68, 73–76]. In the present study, three regiospecific RdOMTs, RdOMT3, RdOMT10, and RdOMT12, were applied in the biotechnological production of methoxyflavonoids. *E. coli* cells harboring RdOMTs carried out the regiospecific methylation of isorhamnetin and naringenin and produced 3-*O*-methylisorhamnetin and isosakuranetin as much as the added substrates, respectively. The other examined flavonoids were also successfully bioconverted by RdOMT-transformed *E. coli* cells to the respective methoxyflavonoids, with a higher than 64% yield. These findings demonstrate that the *E. coli* cells harboring RdOMTs are promising tools for the regiospecific production of valuable methoxyflavonoids.

Abbreviations

OMT	<i>O</i> -Methyltransferase
FOMT	Flavonoid <i>O</i> -methyltransferase
SAM	S-adenosyl L-methionine
RdOMT	<i>R. delavayi</i> <i>O</i> -Methyltransferase
RPGD	Rhododendron Plant Genome Database
LB	Luria–Bertani
IPTG	Isopropyl β-D-thiogalactopyranoside
HPLC	High-performance liquid chromatography
LC–MS	Liquid chromatography-mass spectrometry

HMBC	Heteronuclear multiple bond correlation
COMT	Caffeic acid <i>O</i> -methyltransferase
F3'OMT	Flavonoid 3'- <i>O</i> -methyltransferase
F3'/5'OMT	Flavonoid 3'/5'- <i>O</i> -methyltransferase
F3OMT	Flavonoid 3- <i>O</i> -methyltransferase
F4'OMT	Flavonoid 4'- <i>O</i> -methyltransferase

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13765-024-00918-2>.

Supplementary Material 1. Figure S1. Evolutionary analysis of RdOMTs and other functional OMTs. Figure S2. Purification of the recombinant RdOMT2 (a), RdOMT4 (b), RdOMT5 (c), RdOMT6 (d), RdOMT8 (e), and RdOMT9 (f). Figure S3. Multiple alignments of three active RdOMTs and other functional FOMTs. Figure S4. Identification of the methylated products of the RdOMT3 reactions with flavonoid substrates by LC–MS analysis. Figure S5. HPLC analysis of the RdOMT3 reaction with loricitrin. Figure S6. Some HMBC correlations observed in 3-*O*-methylkaempferol (a), 3-*O*-methylquercetin (b), and 3-*O*-methylisorhamnetin (c) produced from kaempferol, quercetin, and isorhamnetin by RdOMT10, respectively. Figure S7. Identification of the methylated products of the RdOMT12 reactions with flavonoid substrates by LC–MS analysis.

Supplementary Material 2. Table S1. Nucleotide sequences of RdOMTs.

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Author contributions

KL and MHC performed the experiments and wrote the manuscript. SHB and SWL analyzed the data and reviewed the manuscript. SWL and MHC conceived the study. All authors read and approved the final manuscript.

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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 supplementary information files].

Declarations

Competing interests

The authors declare that they have no competing interests.

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