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Flavonoid 3′-*O*-methyltransferase from rice: cDNA cloning, characterization and functional expression

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Abstract

Plant *O*-methyltransferases (OMTs) are known to be involved in methylation of plant secondary metabolites, especially phenylpropanoid and flavonoid compounds. An OMT, *ROMT-9*, was cloned and characterized from rice using a reverse transcriptase polymerase chain reaction (RT-PCR). The blast results for ROMT-9 showed a 73% identity with caffeic acid OMTs from maize and *Triticum aestivum*. ROMT-9 was expressed in *Escherichia coli* and its recombinant protein was purified using affinity chromatography. It was then tested for its ability to transfer the methyl group of *S*-adenosyl-L-methionine to the flavonoid substrates, eriodictyol, luteolin, quercetin, and taxifolin, all of which have a 3′-hydroxyl functional group. The reaction products were analyzed using TLC, HPLC, HPLC/MS, and NMR spectroscopy. The NMR analysis showed that ROMT-9 transferred the methyl group specifically to the 3′-hydroxyl group of quercetin, resulting in the formation of its methoxy derivative. Furthermore, ROMT-9 converted flavonoids containing the 3′-hydroxy functional group such as eriodictyol, luteolin, quercetin and taxifolin into the corresponding methoxy derivatives, suggesting that ROMT-9 is an OMT with strict specificity for the 3′-hydroxy group of flavonoids.

Keywords: Rice; *Oryza sativa*; Gramineae; *O*-methyltransferase; Flavonoids; 3′-*O*-methylation

1. Introduction

Plants produce many kinds of secondary metabolite that are rarely found in other organisms. Flavonoids and phenylpropanoids are typical examples of natural plant products with great structural diversity, which results from a variety of modification reactions that occur once the backbone of these compounds is synthesized. Cytochrome P450s (P450s), glycosyltransferases (GTs), and *O*-methyltransferases (OMTs) have been shown to be involved in the biosynthesis of these compounds. The genes encoding these products sometimes exist as gene families that have been made accessible through various plant genome projects (Ausubel, 2002). Among the several modification reactions, *O*-methylation that is mediated by OMTs transfers a methyl group, S-adenosyl-L-methionine (AdoMet), to the hydroxyl group of a methyl-acceptor molecule (Ibrahim et al., 1998; Ibrahim and Muzac, 2000). Plant OMTs have been functionally characterized from several plants. The most commonly studied OMTs are those that utilize phenylpropanoid and flavonoid compounds as substrates. The *O*-methylation of caffeoyl CoA provides the guaiacyl building blocks for the biosynthesis of lignin, while that of flavonoids is known to reduce the chemical reactivity of phenolic hydroxyl groups and to increase antimicrobial activity (Luckner, 1990; Ibrahim et al., 1998). Several OMTs are known to methylate flavonoids (Ibrahim and Muzac, 2000). Most of these enzymes are highly specific, as has been demonstrated with both purified native and recombinant proteins. Sequences of a myriad of OMT genes from genome projects from various plants have been

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deposited in the GenBank and The Institute of Genome Research (TIGR) databases. These sequences are annotated as OMTs based on primary sequence similarities. However, several previous studies showed that differences of only a few amino acids could generate differences in substrate preferences and that 85%, or higher, sequence identity among some OMTs might bias the prediction of their actual substrates (Gauthier et al., 1998; Frick and Kutchan, 1999; Wang and Pichersky, 1999; Schröder et al., 2002). This suggests that the biochemical analysis of each gene is required for the functional characterization of individual OMTs.

Rice (*Oryza sativa*) is a major global foodstuff and a good model crop plant. Most of its genome sequence is now available (Goff et al., 2002; Yu et al., 2002) and functional analysis of individual rice genes is ongoing. A variety of OMTs are found in rice, although none of them has yet been functionally characterized with the exception of a flavonoid 7-OMT that is involved in sakuranetin biosynthesis (Rakwal et al., 2000). In order to determine the function of such OMTs, we cloned an *ROMT-9* from rice and expressed it in *Escherichia coli*. Here, we report the molecular analysis and functional characterization of *ROMT-9* from rice.

2. Results and discussion

2.1. Isolation and expression of ROMT-9

The rice genome was searched using a class 2 OMT domain (SGLSSLVDVGGGTGALAAAIVRAYPHLKGIVFDLPHVVADAPSADRVEFVGGD) and 19 unique OMT genes were found. Of these, 16 OMTs showed homology with caffeic acid OMT (COMT) and three with caffeoyl CoA OMT (CCoAOMT). cDNAs corresponding to the 19 OMT genes were cloned by RT-PCR using cDNA synthesized from a 2-week-old whole rice plant and were sequenced. Among these, 15 OMTs were expressed in *E. coli* and the ROMT-9 (GenBank accession number 29893141) was the most well-expressed protein. Thus, *ROMT-9* was selected for further investigation. Sequence analysis of *ROMT-9* showed an open reading frame (ORF) of 1164 base pairs encoding a 39.7-kDa protein with a calculated isoelectric point of 5.14. The predicted protein sequence had a 73% identity with COMTs from *Zea mays* and *Triticum aestivum* and a 71% similarity with COMTs from *Saccharum officinarum* and *Lolium perenne*.

As OMTs utilize AdoMet as the methyl group donor, the AdoMet binding sites in OMTs are well conserved. For instance, eight amino-acid residues involved in the AdoMet binding site were similar among chalcone *O*-methyltransferase (*ChOMT*), isoflavone *O*-methyltransferase (*IOMT*) and COMT although these enzymes utilize different substrates (Zubieta et al., 2001). The ROMT-9 AdoMet binding site was also predicted by comparing the ROMT-9 sequence with other OMTs. As shown in Fig. 1, the AdoMet binding site of the ROMT-9 is similar in the three OMTs. The substrate binding site was also predicted based on the determined structure of COMT and IOMT (Zubieta et al., 2001). ROMT-9 has the same binding site as COMT except for one amino-acid difference, Val321 instead of Ile 316 (Fig. 1).

ROMT-9 was expressed in root, stem and leaf tissue, although its expression is higher in the stems and roots than in the leaves (data not shown).

To investigate the biological function of ROMT-9, its open reading frame was cloned into pET 15b vector, expressed as a His-tag fusion protein, and purified to near homogeneity, as indicated by SDS–PAGE. The molecular weight of the recombinant ROMT9 corresponded to the combined molecular weight of ROMT-9 (ca. 39.7 kDa) and that of six histidine residues (ca. 0.09 kDa) (Fig. 2).

2.2. Determination of substrate

The purified ROMT-9 was incubated with several putative substrates namely apigenin, caffeic acid (**6**), catechin (**7**), daidzein, eriodictyol (**4**), ferulic acid, 5-OH ferulic acid, genistein, gossypetin, luteolin (**5**), myricetin (**3**), naringenin, orcinol, quercetin (**1**) and taxifolin (**2**) in order to determine its substrate specificity range (Table 1). Analysis of the metabolites by both TLC and HPLC showed that catechin (**1**), eriodictyol (**4**), luteolin (**5**), myricetin (**3**), quercetin (**1**) and taxifolin (**2**) which all possess a 3′-OH functional group, acted as methyl acceptors, and each substrate produced a new metabolite. However, the flavonoids that lacked a 3′-OH group did not give any reaction product. This result indicates that the methylation position is likely to occur on the 3′-OH group. Using luteolin (**5**), quercetin (**1**), and eriodictyol (**4**) as substrates, the enzyme reaction products were analyzed by HPLC. The reaction from each substrate generated a new peak that exhibited a retention time that differed from that of the substrate. Luteolin (**5**) gave a product with a similar retention time (14.1 min) and UV spectrum to those of an authentic sample of 3′-methylated luteolin (Fig. 3). In addition, both the retention times and UV spectra of the reaction products of both quercetin (**1**) and eriodictyol (**4**) were indistinguishable from those of their authentic 3′-methylated derivatives (data not shown), indicating that ROMT-9 catalyzes methyl group transfers preferentially to the 3′-position of flavonoids. Quercetin (**1**) (a flavonol) and luteolin (**5**) (a flavone), which both possess a C-ring double bond, were the best substrates among the several compounds tested (Table 1), indicating that the presence/absence of the 3-OH group has little, or no, influence on ROMT-9 reactivity towards these substrates. Furthermore, ROMT-9 could transfer only one methyl group into the 3′ hydroxyl group of myricetin (**3**) and could not produce further methylation in other positions. This is in contrast with the *Arabidopsis thaliana AtOMT1*, which exhibits a preference for quercetin (**1**) over luteolin (**5**) as a substrate (Muzac et al., 2000) and the *Catharanthus roseus CrOMT2*, which catalyzes the sequential

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COMT        1 -----MGSTGETQITPTHISDEEANLFAMQLASSASVLPMILKSALELDLLEIIAKAGP--
AtOMT-1     1 -----MGSTAETQLTPVQVTDDEAALFAMQLASSASVLPMALKSALELDLLEIMAKN----
ROMT-9      1 --------MGSTAADMAAAADEEACMYALQLASSSILPMTLKNAIELGLLETLQSAAVAG
ChOMT       1 MGNSYITKEDNQISATSEQTEDSACLSAMVLTTNLVYPAVLNAAIDLNLFEIIAKATPP-
IOMT        1 --------MASSINGRKPSEIFKAQALLYKHIYAFIDSMSLKWAVGMNIPNIIHNHG---

COMT       54 ----GAQISPIEIASQLPTTN--PDAPVMLDRMLRLLACYIILTCSVRTQQDGKVQRLYG
AtOMT-1    52 ----GSPMSPTEIASKLPTKN--PEAPVMLDRILRLLTSYSVLTCSNRKLSGDGVERIYG
ROMT-9     53 GGGKAALLTPAEVADKLPSKAN-PAAADMVDRMLRLLASYNVVRCEMEEGADGKLSRRYA
ChOMT      60 ----GAFMSPSEIASKLPASTQHSDLPNRLDRMLRLLASYSVLTSTTRTIEDGGAERVYG
IOMT       50 -----KPISLSNLVSILQVPS---SKIGNVRRLMRYLAHNGFFEIITK--E----EESYA

COMT      108 LATVAKYLVKNEDGVSISALNLMNQDKVLMESWYHLKDAVLDGGIPFNK-AYGMTAFEYH
AtOMT-1   106 LGPVCKYLTKNEDGVSIAALCLMNQDKVLMESWYHLKDAILDGGIPFNK-AYGMSAFEYH
ROMT-9    112 AAPVCKWLTPNEDGVSMAALALMNQDKVLMESWYYLKDAVLDGGIPFNK-AYGMTAFEYH
ChOMT     116 LSMVGKYLVPDESRGYLASFTTPLCYPALLQVWMNFKEAVVDEDIDLFKNVHGVTKYEFM
IOMT       96 LTVASELLVRGSD-LCLAPMVECVLDPTLSGSYHELKKWIYEEDLTLFGVTLGSGFWDFL

COMT      167 GTDPRFNKVFNKGMSDHSTITMKKILETYTGFEGLKSLVDVGGGTGAVINTIVSKYPTIK
AtOMT-1   165 GTDPRFNKVFNNGMSNHSTITMKKILETYKGFEGLTSLVDVGGGIGATLKMIVSKYPNLK
ROMT-9    171 GTDARFNRVFNEGMKNHSVIITKKLLDLYTGFDAASTVVDVGGGVGATVAAVVSRHPHIR
ChOMT     176 GKDKKMNQIFNKSMVDVCATEMKRMLEIYTGFEGISTLVDVGGGSGRNLELIISKYPLIK
IOMT      155 DKNPEFNTSFNDAMASDSKLINLALRDCDFVFDGLESIVDVGGGTGTTAKIICETFPKLK

COMT      227 GINFDLPHVIEDAPSYPGVEHVGGDMFVSIPKA-DAVFMKWICHDWSDEHCLKFLKNCYE
AtOMT-1   225 GINFNLPHVIEDAPSHPGIEHVGGDMFVSVPKG-DAIFMKWICHDWSDEHCVKFLKNCYE
ROMT-9    231 GINYDLPHVISEAPPFPGVEHVGGDMFASVPRGGDAILMKWILHDWSDEHCARLLKNCYD
ChOMT     236 GINFDLPQVIENAPPLSGIEHVGGDMFASVPQG-DAMILKAVCHNWSDEKCIEFLSNCHK
IOMT      215 CIVFDRPQVVENLSGSNNLTYVGGDMFTSIPNA-DAVLLKYILHNWTDKDCLRILKKCKE

COMT      286 ALPDNGK---VIVAECILPVAPDSSLATKGVVHIDVIMLAHNPGGKERTQKEFEDLAKGA
AtOMT-1   284 SLPEDGK---VILAECILPETPDSSLSTKQVVHVDCIMLAHNPGGKERTEKEFEALAKAS
ROMT-9    291 ALPEHGK---VVVVECVLPESSDATAREQGVFHVDMIMLAHNPGGKERYEREFRELARAA
ChOMT     295 ALSPNGK---VIIVEFILPEEPNTSEESKLVSTQDNLMFITLVGGRERTEKQYEKLSKLS
IOMT      274 AVTNDGKRGKVTIIDMVINEKKDENQVTQIKLLMDVNMACLLNGKERNEEEWKKLFIEA

COMT      343 GFQGFKVHCNAFNTYIMEFLKKV
AtOMT-1   341 GFKGIKVVCDAFGVNLIELLKKL
ROMT-9    348 GFTGFKATYIYANAWAIEFTK--
ChOMT     351 GFSKFQVACRAFNSLGVMEFYK-
IOMT      332 GFQHYKISPLTGFLSLIEIYP--
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Fig. 1. Sequence alignment of one COMT and four representative plant *O*-methyltransferases. Shown are primary sequences of COMT from alfalfa (AAB46623), AtOMT-1 from *Arabidopsis* (U70424), ROMT-9 from rice, chalcone *O*-methyltransferase (ChOMT; AAB48059) from alfalfa, and isoflavone *O*-methyltransferase (IOMT; AAC49927) from alfalfa. ■: Active site dimer; ■: conserved residues and motifs for AdoMet binding; ■: catalytic residue; ■: active site substrate binding/positioning residue.

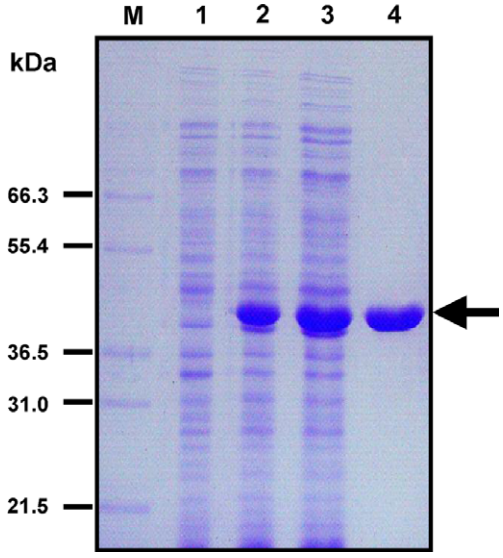


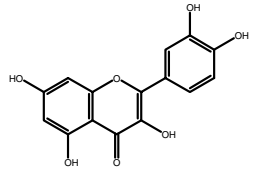
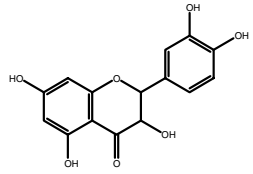
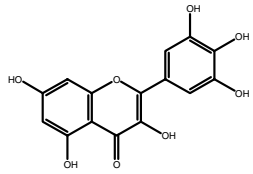
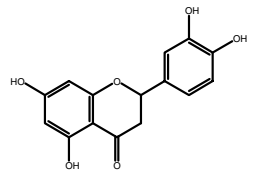
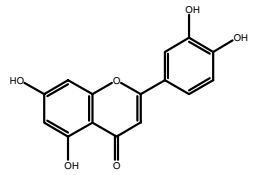
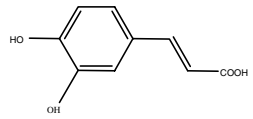
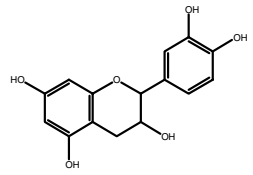
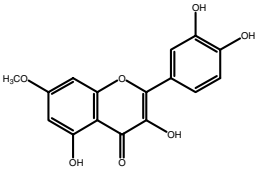
Fig. 2. SDS–PAGE of the expressed recombinant ROMT-9. M, Standard protein markers; 1, *E. coli* lysate before induction; 2, *E. coli* lysate after induction; 3, soluble protein after induction; 4, His-tagged affinity-purified protein.

methylation of the two 3′- and 5′-hydroxyl groups of myricetin (**3**) (Cacace et al., 2003). In addition, ROMT9 could not convert 4′-methylated quercetin (tamarixetin) while it could use 7-methylated quercetin (**8**) (rhamnetin (**8**)) as effectively as quercetin (**1**), indicating that the position of the methyl group is critical for the methylation of ROMT-9. It also contrasts with pFOMT3′ from *Chrysosplenium americanum*, which prefers trimethylated quercetin to un- or mono-methylated quercetin (Gauthier et al., 1996). Flavonoids, such as taxifolin (**2**) and eriodictyol (**4**), were less effective methyl acceptors (Table 1), possibly due to the saturation of the C-ring. The fact that there was no significant activity with caffeic acid (**6**) and 5-OH ferulic acid as substrates indicates that ROMT-9 is not involved in the methylation of lignin precursors.

The kinetic parameters K_m and V_{max} for eriodictyol (**4**), luteolin (**5**), and quercetin (**1**) were determined using Lineweaver–Burk plots. As predicted from their relative enzyme activities (Table 2), quercetin (**1**) appears to be the best methyl acceptor, based on its turnover value (K_{cat}/K_m), followed by luteolin and eriodictyol (Table 2).

A compound was found in the acid hydrolyzates of rice leaves that co-chromatographed with and exhibited a similar UV spectrum to an authentic sample of chrysoeriol, 3′-methylated luteolin (data not shown). Therefore, the biological function of ROMT-9 probably involves the transfer of a methyl group to luteolin (**5**). By contrast, quercetin (**1**) and kaempferol, which are ubiquitous in other species such

Table 1
Relative activity of ROMT9 with various substrates

Substrate	Structure	Retention times (S/P; min)[a]	Conversion rate (%)
Quercetin **1**		11.5/14.6	100[b]
Taxifolin **2**		7.4/9.6	71
Myricetin **3**		9.4/14.7	94
Eriodictyol **4**		11.4/14.3	92
Luteolin **5**		11.3/14.1	96
Caffeic acid **6**		5.1/7.2	15
Catechin **7**		3.8/5.3	15
Rhamnetin **8**		17.4/20.3	88

[a] S, substrate (70 μM was used); P, product.
[b] 100% is equivalent to 1250 pkat/mg.

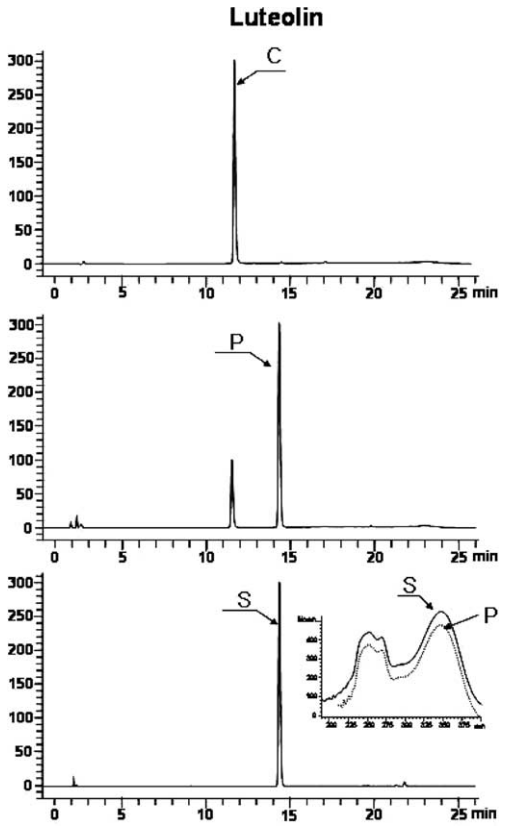


Fig. 3. HPLC elution profiles and UV spectra for the reaction product from luteolin (**5**) (C: substrate, P: reaction product, S: methylated authentic compound).

as *Arabidopsis* and soybean (Graham, 1991, 1998) were not detected in rice leaf extract.

2.3. Determination of the methylation position in quercetin (*1*) by NMR spectroscopy

To verify the methylation position of quercetin (**1**) and to determine the regioselectivity of ROMT-9, quercetin (**1**) as substrate and its reaction product (9) were subjected to NMR spectroscopic analysis. The ^{1}H and ^{13}C NMR spectra of the reaction product gave new peaks at 3.83 and 55.9 ppm, respectively, which were not observed in quercetin (**1**) itself. Comparing the ^{1}H and ^{13}C NMR spectra of quercetin (**1**) with those of its reaction product (Agrawal, 1989; Harborne, 1994), all of the ^{1}H and ^{13}C chemical shifts could be readily assigned. As the nOe cross peak between H-2′ at 7.75 ppm and the methyl proton at 3.83 ppm was observed in the NOESY spectrum, the methylated position was identified as the 3′-hydroxyl group. In order to clarify this result, HMBC analysis was performed. Based on its interpretation (Fig. 4), C-3′ was long-range coupled to the methyl proton, so that the methylated position appear to be the 3′-hydroxyl group. The ^{1}H NMR spectrum of the reaction product of quercetin (**1**) (Fig. 5(a)) was compared with that of an authentic sample of isorhamnetin, 3′-methyl quercetin (Fig. 5(b)). As a result, ROMT-9 was shown to transfer a methyl group to the 3′-OH quercetin and to be a 3′-*O*-methyltransferase. The assignments of the ^{1}H and ^{13}C NMR spectroscopic data of the reaction product of quercetin (**1**) are listed in Table 3.

ROMT-9 gene turns out to be a flavonoid 3′-*O*-methyltransferase which prefers flavonol or flavone to flavanone. Flavonoid 3′-methylation is one of the most common modification reactions found in nature. ROMT9 is the first flavonoid 3′-*O*-methyltransferase cloned in rice as far as we know.

3. Experimental

3.1. Chemicals

Flavonoids were purchased from Indofinechemicals (Somerville, NJ, USA). HPLC-grade organic solvents were purchased from Duksan Co. (Ansan, Korea).

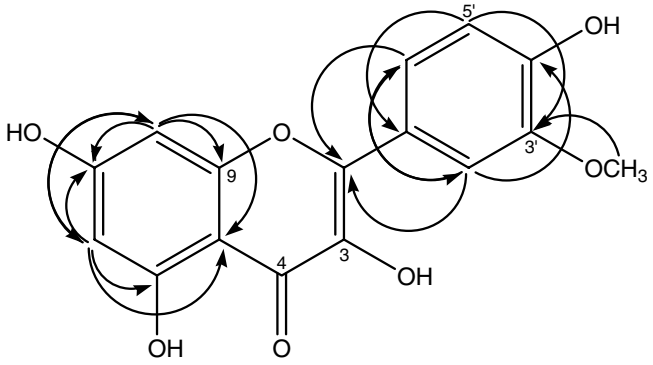


Fig. 4. The proton–carbon long-ranged couplings obtained from the HMBC interpretation of the reaction product (9) of quercetin (**1**) produced by the purified recombinant protein ROMT-9.

Table 2
Substrate specificity of purified recombinant protein ROMT-9

Substrate	Relative activity (% of control)	K_m (μM)	V_{max} (pkat/mg)	V_{max}/K_m	K_{cat}/K_m (μM^{-1} s^{-1})
Quercetin **1**	100	61.8	1250.0	20.2	0.42
Luteolin **5**	96	61.9	833.3	13.5	0.28
Eriodictyol **4**	92	62.0	625.0	10.1	0.21

Enzyme assays were carried out using 1–2 μg of the purified ROMT-9, 10–200 μM of each substrates, and 40 μM of AdoMet.

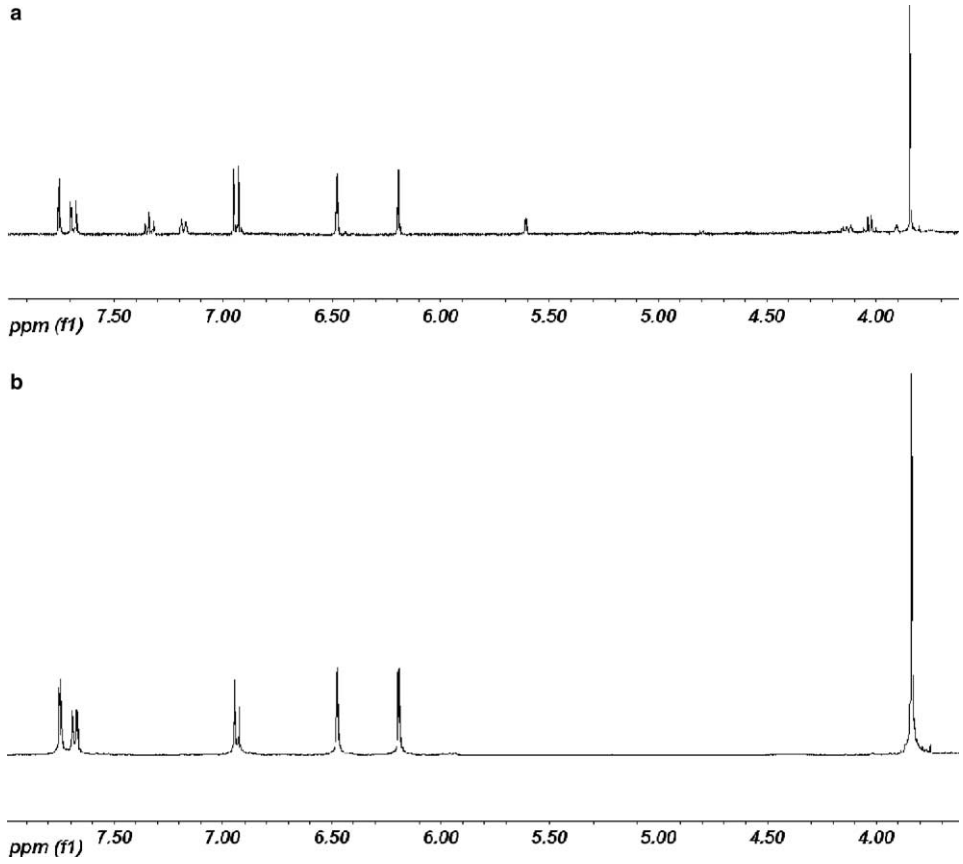
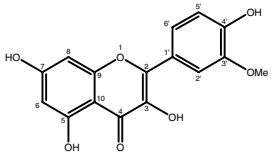


Fig. 5. The ^{1}H NMR spectra of (a) the reaction product of quercetin and (b) the authentic sample of isorhamnetin.

Table 3
The assignments of ^{1}H and ^{13}C NMR spectroscopic data for the product of quercetin **1** generated using purified recombinant protein ROMT-9 and **1a** substrate



Position	δ_H (*J*, Hz)/ppm	δ_C/ppm	Long-ranged couplings by HMBC	nOe cross peaks by NOESY
2	–	146.7	–	–
3	–	135.9	–	–
4	–	175.9	–	–
5	–	160.7	–	–
6	6.18 (*d*, 2.0)	98.3	C-5, C-7, C-8, C-10	–
7	–	164.1	–	–
8	6.46 (*d*, 2.0)	93.7	C-6, C-7′, C-9, C-10	–
9	–	156.3	–	–
10	–	103.1	–	–
1′	–	122.1	–	–
2′	7.75 (*d*, 2.0)	111.8	C-2, C-4′, C-6′	3′-OMe
3′	–	147.5	–	–
4′	–	148.9	–	–
5′	6.93 (*d*, 8.5)	115.6	C-1′, C-3′	H-6′
6′	7.68 (*dd*, 8.5, 2.0)	121.8	C-2, C-2′	H-5′
3′-OMe	3.83 (*s*)	55.9	C-3′	H-2′

3.2. Cloning of ROMT-9

Total RNA from the leaf, root, and stem tissues of 2-week-old rice plants was isolated using a Qiagen RNA isolation kit (Qiagen, Gaithersburg, MD, USA). RT-PCR was used to clone ROMT-9 from rice. cDNA was synthesized in 20 μl reaction mixtures containing 2 μg of total RNA, Omniscript transcriptase (Qiagen, Gaithersburg, MD,

USA), 15 pmol oligo $(dT)_{15}$ and 20U of RNasin (Promega, WI, USA). For the PCR we used Hot start Taq DNA polymerase (Qiagen, Germany) under the following conditions; 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 1.5 min amplification at 72 °C. The primers used were GCTAGCTAGGATGGGTTCTACA as a forward primer (starting 10-bp in front of the start codon) and CGATGGTCGAACACCTTGAT as the reverse primer (ending 44-bp behind the stop codon). The PCR product was subcloned into the pGEMT-easy vector (Promega) and the resulting plasmid was sequenced (GenBank accession number DQ288259).

3.3. Expression of ROMT-9 in Escherichia coli

To construct the expression vector for ROMT-9, its ORF was amplified by PCR with ATCATATGGGTTCTACAGCCGCCGA as the forward primer and ATGGATCCTCGCCAATCGCCTACTTGGA as the reverse primer. The restriction enzyme sites, *Nde*I and *Bam*HI (underlined), were added to facilitate the cloning process. The resulting PCR product was cut with *Nde*I and *Bam*HI, and was subcloned into the corresponding sites of pET15b (Novagen, Madison, WI, USA). The transformant was grown in LB medium containing 50 μg/ml ampicillin. The culture grew until an absorbance of 0.7 at 600 nm was reached. At this point, IPTG was added at a final concentration of 1 mM and the transformant was grown for 5 h at 30 °C. The bacterial cells were then harvested, resuspended in His-tag binding buffer (20 mM NaH_2PO_4, 500 mM NaCl, pH 7.4), and lysed by sonication. The expressed protein was purified with a His-tag affinity column (Amersham Biosciences, USA), and its purity was analyzed by SDS–PAGE.

3.4. Enzyme assay and analysis of reaction product

To determine the ROMT-9 enzymatic activity, a reaction mixture was prepared containing 1–2 μg of the purified recombinant protein, 2 mM DTT, 40 μM AdoMet and 200 μM substrate in 10 mM Tris/HCl buffer (pH 7.5) at a final volume of 500 μl. The reaction mixture was incubated at 37 °C for 1 h, extracted twice with ethylacetate and the organics layer was evaporated to dryness. Flavonoids were also analyzed by HPLC (Palo Alto, CA, USA) using an Agilent 1000 C18 reversed-phase column (Waters, Milford, MA, USA; 4.60×250 mm, 0.6 μm) and a photodiode array detector. For analytical scale, the mobile phase consisted of 50 mM phosphate buffer (pH 3.0) that was programmed as follows: 10% acetonitrile at 0 min, 30% acetonitrile at 10 min, 60% acetonitrile at 40 min, 90% acetonitrile at 45 min and 10% acetonitrile at 50 min. The flow rate was 1 ml/min and UV detection was performed at 270 nm. Quantification of the metabolites and the parent material over time was monitored using HPLC in duplicate experiments. Several different concentrations of each substrate were analyzed with HPLC, and the resulting value was used as a standard for the analysis of the remaining reaction product after enzymatic conversion of substrates.

3.5. Liquid chromatography/mass spectrometry

Liquid chromatography (LC) was performed as described above. Mass spectrometry (MS) was carried out by coupling an HP 1100 system to a Quattro LC triple quadruple tandem mass spectrometer (Micromass, Manchester, UK) with an electrospray ionization (ESI^+) mode. Full scans were acquired in positive ion modes. The source temperature, desolvation temperature, cone voltage, and capillary voltage were kept at 110 °C, 180 °C, 28 V, and 3.88 kV, respectively. An electron multiplier voltage of 640 V was used. The nebulizer gas and desolvation gas were ultra-pure nitrogen set at 81 l/h, 300 l/h.

3.6. Nuclear magnetic resonance spectroscopy

The nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance 400 instrument (9.4 T, Karlsruhe, Germany) in DMSO-d_6. For the ^1H NMR experiment, 32 transients were acquired with a 1-s relaxation delay using 32K data points and the 90° pulse was 9.8 μs, with a spectral width of 4500 Hz. The ^{13}C NMR experiments were carried out with spectral width of 22,700 Hz using 64K data points, and its 90° pulse was 10.3 μs. Two-dimensional spectra were acquired with 2048 data points in t2 and 256 in t1 increments. The COSY and HMBC spectra were collected using the magnitude method, and the TOCSY, NOESY, and HMQC spectra, were produce with the phase sensitive mode. The data were processed using xwinnmr software provided by Bruker.

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References

Agrawal, P.K. (Ed.), 1989. Carbon-13 NMR of Flavonoids. Elsevier, New York, pp. 152–155.

Ausubel, F.M., 2002. Summaries of National Science Foundation-Sponsored Arabidopsis 2010 Projects and National Science Foundation-Sponsored Plant Genome Projects that are generating Arabidopsis Resources for the Community. Plant Physiol. 129, 394–437.

Cacace, S., Schröder, G., Wehinger, E., Starck, D., Schmidt, J., Schröder, J., 2003. A flavonol *O*-methyltransferase from *Catharanthus roseus* performing two sequential methylations. Phytochemistry 62, 127–137.

Frick, S., Kutchan, T.M., 1999. Molecular cloning and functional expression of *O*-methyltransferases common to isoquinoline alkaloid and phenylpropanoid biosynthesis. Plant J. 17, 329–339.

Gauthier, A., Gulick, P.J., Ibrahim, R.K., 1996. cDNA cloning and characterization of a 3′/5′-*O*-methyltransferase for partially methylated flavonols from *Chrysosplenium americanum*. Plant Mol. Biol. 32, 1163–1169.

Gauthier, A., Gulick, P.J., Ibrahim, R.K., 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.

Goff, S.A., Ricke, D., Lan, T.H., Presting, G., et al., 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp. japonica). Science 296, 92–100.

Graham, T.L., 1991. Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiol. 95, 594–603.

Graham, T.L., 1998. Flavonoid and flavonol glycoside metabolism in *Arabidopsis*. Plant Physiol. Biochem. 36, 135–144.

Harborne, J.B. (Ed.), 1994. The Flavonoids. Advances in Research. Chapman & Hall, London, pp. 452–453.

Ibrahim, R.K., Bruneau, A., Bantignies, B., 1998. Plant *O*-methyltransferase: molecular analysis, common signature and classification. Plant Mol. Biol. 36, 1–10.

Ibrahim, R.K., Muzac, I., 2000. The methyltransferase gene superfamily: a tree with multiple branches. In: Romeo, J.T., Ibrahim, R.K., Varin, L., De Luca, V. (Eds.), Evolution of Metabolic Pathways. Pergamon Press, Amsterdam, pp. 349–384.

Luckner, M., 1990. Secondary Metabolism in Microorganisms, Plants and Animals, third ed. Springer-Verlag, New York.

Muzac, I., Wang, J., Anzellotti, D., Zhang, H., Ibrahim, R.K., 2000. Functional expression of an Arabidopsis cDNA clone encoding a flavonol 3′-*O*-methyltransferase and characterization of the gene product. Arch. Biochem. Biophys. 375, 385–388.

Rakwal, R., Agrawal, G.K., Yonekura, M., Kodama, O., 2000. Naringenin 7-*O*-methyltransferase involved in the biosynthesis of the flavanone phytoalexin sakuranetin from rice (*Oryza sativa* L.). Plant Sci. 155, 213–221.

Schröder, G., Wehinger, E., Schröder, J., 2002. Predicting the substrates of cloned plant *O*-methyltransferases. Phytochemistry 59, 1–8.

Wang, J., Pichersky, E., 1999. Identification of specific residues involved in substrate discrimination in two plant *O*-methyltransferases. Arch. Biochem. Biophys. 368, 172–180.

Yu, J., Hu, S., Wang, J., Wong, G.K., et al., 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp. indica). Science 296, 79–92.

Zubieta, C., He, X.Z., Dixon, R.A., Noel, J.P., 2001. Structures of two natural product methyltransferases reveal the basis for substrate specificity in plant *O*-methyltransferases. Nat. Struct. Biol. 8, 271–279.