Influence of Substrate Binding Residues on the Substrate Scope and Regioselectivity of a Plant O-Methyltransferase against Flavonoids

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Introduction

Flavonoids are a large group of natural polyphenols and secondary metabolites from plants. They attract a lot of attention due to their nutritional, health-beneficial and pharmacological properties including free radical-scavenging antioxidative activities, anti-inflammatory, antimicrobial and anticancer activities. Flavonoid methylation is usually achieved by a specific group of plant O-methyltransferases (OMTs) which typically exhibit high substrate specificity. Here we investigated the effect of several residues in the binding pocket of the Clarkia breweri isoeugenol OMT on the substrate scope and regioselectivity against flavonoids. The mutation T133M, identified as reported in our previous publication, increased the activity of the enzyme against several flavonoids, namely eriodictyol, naringenin, luteolin, quercetin and even the iso-flavonoid genistein, while a reduced set of amino acids at positions 322 and 326 affected both, the activity and the regioselectivity of the methyltransferase. On the basis of this work, methylated flavonoids that are rare in nature were produced in high purity.

Methylation of free hydroxyl groups is an important modification for flavonoids. It not only greatly increases absorption and oral bioavailability of flavonoids, but also brings new biological activities. Flavonoid methylation is usually achieved by a specific group of plant O-methyltransferases (OMTs) which typically exhibit high substrate specificity. Here we investigated the effect of several residues in the binding pocket of the Clarkia breweri isoeugenol OMT on the substrate scope and regioselectivity against flavonoids. The mutation T133M, identified as reported in our previous publication, increased the activity of the enzyme against several flavonoids, namely eriodictyol, naringenin, luteolin, quercetin and even the iso-flavonoid genistein, while a reduced set of amino acids at positions 322 and 326 affected both, the activity and the regioselectivity of the methyltransferase. On the basis of this work, methylated flavonoids that are rare in nature were produced in high purity.
from soy bean (Glycine max) and SaOMT2 from Streptomyces avermitilis were discovered to show both flavonoids and isoflavonoids methylation activities.[9] The crystal structure of an IOMT with isoformononetin (4′-hydroxy-7-methoxyisoflavone) in the active site shows that some residues are critical for the stabilization of isoflavonoids.[10] However, no crystal structure of FOMT is yet available.

In order to study the substrate discrimination between the plant OMTs, we compared the substrate binding residues of POMT, FOMT and IOMT with different substrate preferences and regioselectivities. Based on the differences of these residues, we have designed and constructed mutants based on the isoformononetin (4-vinyl-3′,7-dihydroxyisoflavone) which has been obtained in a catalytic reaction of the enzyme from Clarkia breweri including the mutation T133M (i.e.OMT_T133M). In a previous work of ours,[11] we identified this variant to have expanded substrate scope and altered regioselectivity against phenolic compounds. Thus, it was selected as the starting point for this study. We discovered that some mutants specifically methylated the 3′-OH of the tested flavonoids and others further methylated the 4′-OH, producing dimethylated flavonoids. The tested mutants also brought activities and different regioselectivities to the isoflavonoid genistein.

**Results and Discussion**

**Substrate binding residues of different plant OMTs**

Methylation reactions mostly take place at the 7-, 3′- and 4′-hydroxyl groups of flavonoids, the 7- and 4′-hydroxyl groups of isoflavonoids and the 3- and 4-hydroxyl groups of phenylpropanoids. In order to discover the factors determining the substrate discrimination of plant OMTs, we chose 21 plant OMTs from different plant species with different substrate preferences and regioselectivities for comparison. Sequence alignment shows that the sequences are extremely diverse between different plant OMTs, with only 46.6% identity (Figure 2, Figure S1). Since several IOMT crystal structures have been resolved but no FOMT structure is available, we chose MeSa-7/4′-IOMT which has been obtained in a catalytic conformation (PDB code: 1FP2) and investigated based on it the substrate-enzyme interactions.[11] The ligand isoformononetin, the 7-methylated product of the isoflavonone daidzein, is situated in the enzyme active site and well-stabilized by multiple interactions. Residues critical for substrate binding are highlighted in Figure 1. Met183 and Met322 constrain the aromatic A-ring and help positioning the 7-hydroxyl group to the catalytic residue His272 and SAM. These two residues are conserved throughout the plant OMT superfamily. Zubieta et al. suggested that the interaction of the ketone group in the C-ring is stabilized by the amide side chain of Asn322, a residue that is only conserved among the 7-IOMTs. Other OMTs rather have middle size hydrophobic residues, namely Ile, Val or Met, at this position. Leu326 also interacts with the C-ring of isoformononetin, but it locates closer to the ether oxygen. Residues at this position are quite different between the selective OMTs. They are either the hydrophobic Leu, Val or Met, or the basic residues Arg or His. In the absence of Asn322, the basic residues might play an important role in stabilizing the C-ring ketone group. The accommodation of the isoflavone B-ring is achieved by Cys133 and Val134. Although these two residues in other plant OMTs are quite diverse, they mostly have Gly/Leu/Met and Ala/Val/Asn at these two positions, respectively. These substrate binding residues bring proper binding patterns to their preferred substrates and thus determine substrate specificity and regioselectivity of different

![Figure 1](image1.png)  
*Figure 1. Structures of the flavonones eriodictyol and naringenin, the flavone luteolin, the flavonol quercetin and the isoflavone genistein. Ring assignment and backbone atoms numbering are shown in the structure of eriodictyol.*

![Figure 2](image2.png)  
*Figure 2. Part of the sequence alignment of plant flavonoid OMTs (FOMTs), isoflavonoid OMTs (IOMTs) and phenylpropanoid OMTs (POMTs) performed with Geneious 10.0.2 (the whole sequence alignment is shown in Figure S1). Residues involved in substrate binding are highlighted in blue. Numbering of residues is based on the sequence of the Clarkia breweri isoflavonoid OMT (i.e.OMT). OMTs are named by their original organisms and regioselectivity. MePi, Mentha piperita; MeTr, Medicago truncatulaca; OrSa, Oryza sativa; ArTh, Arabidopsis thaliana; CaRo, Catharanthus roseus; GlEc, Glycine max; CaAr, Cicer arietinum; MeSa, Medicago sativa; GlMa, Glycyrrhiza echinate; CiAr, Cicer arietinum; LoJa, Lotus japonicus; CiBr, Clarkia breweri; LoPe, Lolium perenne. MeTr_7-FOMT is a putative IOMT but it has higher preference against naringenin (flavonone) than isoflavonoids.*
plant OMTs. In order to investigate the influence of these residues, we constructed mutants L322H/N/M and Y326H/R/L using the leOMT-T133M variant as template. leOMT is a phenyl-propanoid 4-OMT isolated from *Clarkia breweri* and the variant T133M has been proved to expand the substrate scope and to enhance the regioselectivity.[11]

### Substrate scope and enzyme activities

Flavonoids are classified into flavane, flavanone, flavone and flavonol, while isoflavonoids are divided into isoflavone and isoflavonane, depending on their structures. We have chosen several commonly known compounds eriodictyol and naringenin (flavanones), luteolin (flavone), quercetin (flavonol) and genistein (isoflavone) as substrates (Figure 1). Since optically pure flavanones will racemize in aqueous solution, we used racemic eriodictyol and naringenin as substrates and obtained racemic products.[12] Activities of the wild type leOMT and designed mutants were tested against these substrates. In each reaction, a molar excess of SAM and 25% (v/v) *Escherichia coli* (E. coli) cell lysate, which contains S-adenosyl-L-homocysteine (SAH) nucleosidase, were provided in order to increase the yield.[13] Products were confirmed by either comparing their retention times on HPLC to commercial standards or structurally characterized via NMR and MS. Catalytic performance of the mutants are presented in area percentages calculated by peak area of both substrates and products measured by HPLC (Figure 3, Figure S2).

It needs to be stated that in an initial screening performed, the leOMT_T133M exhibited an expanded substrate scope compared to the wild type (converting naringenin and genistein), while it also exhibited altered regioselectivity. For this reason, the variant leOMT_T133M was selected as the template for the designed mutations.

Although leOMT is a phenylpropanoid OMT, it shows high activities against the flavonoids eriodictyol, luteolin and quercetin and is highly regiospecific to the 3'-hydroxy group. In the absence of the 3'-hydroxy group, the wild-type leOMT displays very low activity, as seen against naringenin. The variant T133M has a universal effect of increasing the enzymatic activity against these substrates. Moreover, this variant further methylates the 4'-hydroxy groups, producing both 3',4'-dimethylated products. Mutants T133M/Y326H and T133M/Y326L further enabled increased production of 3',4'-dimethylated eriodictyol, luteolin and quercetin. On the other hand, the variant T133M/L322N retains the regiospecificity against the 3'-hydroxy group of all tested flavonoids and so does the mutant T133M/Y326R towards luteolin and quercetin. It is interesting to note that the T133M, T133M/L322N as well as other variants of leOMT, instead of methylating the 4'-OH of naringenin, they methylate the 7-OH. We speculate that the product 2 from naringenin, further produced by variant T133M/Y326L after 48 h, could be another methylated product or further methylation on the methylated naringenin has occurred.

The wild-type leOMT displayed very minor activity towards the isoflavone genistein and the mutants showed different regioselectivity. While T133M, T133M/L322H (or N or M-substitutions) and T133M/Y326R exhibited higher selectivity against the 4'-hydroxy group, T133M/Y326H and T133M/Y326L also produced the 7-methylated genistein. Product 3 was produced by mutants which displayed both 7- and 4'-methylation activities especially by variant T133M/Y326L after 48 h. According to the RP-HPLC chromatograms, it was eluted later than the single methylated genistein (Figure S2). Therefore, we assume that this is the 7,4'-dimethylated genistein.

Interestingly, both naringenin and genistein missing the 3'-OH group lead to 7-methylation, but when the B-ring shifts from position 2 to position 3 of the C-ring, the regioselectivity of some mutants shifted from the 7-hydroxyl group to the 4'-hydroxy group.

### Structural analysis

To gain an insight into the structural differences that lead to the different regioselectivity of the variants, we performed *in silico* analysis. As seen in Figure 4A, the wild-type leOMT can accommodate eriodictyol, luteolin and quercetin in an orientation that the methylation of the 3'-position is favored. In all three cases the distance of the oxygen of 3'-hydroxy group to the methyl group to be transferred is between 3.2 and 3.5 Å. It is interesting to note that eriodictyol and luteolin bind in a similar orientation, however, quercetin seems to be a little tilted in comparison to SAM, which brings also the 4'-position in closer proximity to the transfer group (3.3 Å to the 3'-OH and 3.4 Å to the 4'-OH group) and thus the wild-type leOMT can also produce some dimethylated quercetin. In the case of the double mutant T133M/Y326L, the double methylation is increased for all three substrates. As seen in Figure 4B, the double mutation enabled the inverse binding of these three flavonoids in the binding pocket, bringing the 4'-hydroxy group in the proximity of the methyl group of the SAM and thus the double methylation is favored. The reason for this inversely binding seems to be the more hydrophobic character of the introduced Y326L, which cannot accommodate the carbonyl of 5-position.

In the case of genistein, the T133M mutation increased the activity to a detectable level, but the mutations at positions 322 and 326 do not further increase the activity of the enzyme. However, the double mutant T133M/Y326H has a shift of its regioselectivity to position 7. As seen in Figure 4C, the substrate is bound with ring A facing SAM, and the position 7 is closer to the methyl group for the transfer (3.7 Å). It seems that the histidine at position 326 can interact with the hydroxyl group at position 5 of genistein (3.0 Å distance) and thus stabilizes the substrate in this orientation to complete the catalysis.

Naringenin differs from eriodictyol only by the lack of the 3'-hydroxy group. Thus, although it can bind the same way in the active site of the wild type, the B-ring cannot be methylated to a 3'-methoxy derivative and thus the wild type is almost inactive. However, the mutation T133M (and the T133M/L322M mutations) enabled a different binding pattern, where the position 7 of the ring A is accessible to the SAM and the 7-
The binding pattern differs for genistein, for which the enzyme exhibited the same regioselectivity. In the case of variant T133M/L322M (Figure 4D), the reason for this can be the lack of the histidine at position 326 that could interact with the 5-OH, in combination with the methionine in position 322 that pushes away the substrate. As naringenin is not planar, the ring B may cause steric clashes with 322 and thus the substrate binds in a different orientation.

Conclusions

In this study we highlighted the potential of IeOMT, a phenylpropanoid OMT to be engineered to catalyze the methylation of a range of flavonoids and isoflavonoids. Three positions, namely 133, 322 and 326 had a significant impact on the binding of the substrates and variants that were produced with semi-rational design in these positions had altered catalytic activity and/or regioselectivity compared to the wild-type enzyme. These variants have provided access to methylated flavonoids that are rare in nature and may have interesting biological activities due to their methylation patterns.
Experimental Section

Materials

DpnI was purchased from New England Biolabs, Inc. All primers were purchased from Thermo Fisher Scientific, Inc. Competent cells were self-prepared. S-Adenosyl-l-methionine was purchased from J&K Chemical Ltd. Acetic acid, trifluoroacetic acid (TFA) and acetonitrile were purchased from Carl Roth GmbH and VWR chemicals and were of HPLC grade. (±)-Eriodictyol was purchased from Carl Roth GmbH, (±)-naringenin and quercetin were purchased from Sigma, luteolin, genistein and biochanin A (4′-methoxyistein) were purchased from TCI GmbH, prunetin (7-methoxyistein) and sakuranetin (7-methoxynaringenin) were purchased from Extrasynthese. All these chemicals have 95% or higher purity.

Mutagenesis, expression and purification

IeOMT from Clarkia breweri (accession number of protein: O04385.2) and the mutant T133M in pET-21a(+) with His6-tag at the C-terminal were constructed as described in our previous work.\(^{[11]}\) Further mutagenesis were performed following the QuikChange (Stratagene) protocol using pET-21a(+)–T133M as template. Primers are as below:

- L322H fw: 5’-CACACCGATGCCATATGCTGG-3’
- L322M fw: 5’-CACACCGATGCGATGATGCTGG-3’
- L322N fw: 5’-CACCGATGCGAACATGCTGGCGTATAAC-3’
- L322M rv: 5’-CCAGCATCATCGCATCGGTGTG-3’
- L322N rv: 5’-GTTATACGCCAGCATGTTCGCATCGGTG-3’
- Y326H fw: 5’-TGCTGGCGCATAACCCGGGCGGTAAAG-3’
- Y326H rv: 5’-CTTTACCGCCCGGGTTATGCGCCAGCA-3’
- Y326L fw: 5’-TGCTGGCGCTGAACCCGGGCGGTAAAG-3’
- Y326L rv: 5’-CTTTACCGCCCGGGTTCAGCGCCAGCA-3’
- Y326R fw: 5’-TGCTGGCGCGTAACCCGGGCGGTAAAG-3’
- Y326R rv: 5’-CTTTACCGCCCGGGTTACGCGCCAGCA-3’

After PCR, the pET-21a(+)–T133M template was digested by DpnI before transforming into Escherichia coli (E. coli) Top 10 chemically competent cells for plasmid amplification. After sequence confirmation (Eurofins, Germany), the newly constructed mutant plasmids were transformed into E. coli BL21 (DE3) chemically competent cells for protein expression. Expression and purification of IeOMT and all mutants were carried out following the protocols given in our previous work.\(^{[11]}\)

Enzyme assays and HPLC analysis

The reaction mixture consisted of 150 μM flavonoid substrate, 1 mM SAM, 5 mM DTT and 25% (v/v) E. coli BL21 (DE3) cell lysate (not including the MTs of interest), in 50 mM sodium phosphate buffer.
buffer, pH 7.5. Because the S-adenosyl-L-homocysteine (SAH) derived from SAM is a potent inhibitor for MTs, E. coli cell lysate which contains SAH nucleosidase was provided in each reaction in order to reduce the inhibition effect brought by the SAH.\(^{(1)}\) 1.0 mg/mL (24.4 μM) purified enzymes were added into the reaction mixtures to initiate the enzyme reaction. Due to the instability of SAM, the same amount of SAM was supplied again after 24 h. The mixtures were incubated at 28°C with 700 rpm agitation in an Eppendorf Thermomixer. Assays were performed in triplicate and negative controls were performed by replacing purified enzymes with buffer. Samples were taken at 2, 4, 24 and 48 h and an equal volume of acetonitrile was added to quench the reaction. Samples were vortexed vigorously and then centrifuged at full speed for 30 min to remove protein precipitate. 200 μL supernatant was transferred to HPLC sample vial inserts for analysis. HPLC analysis were performed on VWR Hitachi Elite LaChrom system equipped with the Kinex EVO C18 (4.6×250 mm, 5 μm particle size, Phenomenex) reversed-phase column. 0.1% acetic acid and acetonitrile were used for the separation of flavonoid substrates and the corresponding methylated products, with the ratio 68:32 (v/v) for eriodictyol, luteolin and quercetin, and 60:40 (v/v) for naringenin and genistein. Wavelengths for the detections of flavanones (eriodictyol and naringenin), flavone and flavanol (luteolin and quercetin) and isoflavone (genistein) were 280, 260 and 260 nm, respectively. All analyses were performed at a flow rate of 1 mL/min and the column temperature was 35°C. Identification of methylated products were confirmed by comparing their retention times on HPLC to commercial standards. For unknown products, preparative-scale reactions were performed with specific substrates and mutants and the products were isolated and identified by NMR and MS. Area percentages of substrate and each product were calculated to show the estimated yield for each product.

### Biosynthesis, isolation and purification of unknown methylated products

Each preparative-scale reaction started with 30 mg flavonoid substrate (~100 μmol), 80 mg SAM (200 μmol), 20 mg purified enzyme, 0.1% NaCl, and crude E. coli cell lysate in 100 mL sodium phosphate buffer (50 mM, pH 7.5). Purified IeOMT_T133M/Y326R was used for the biosynthesis of the 3'-methylated products of eriodictyol, luteolin and quercetin. The accession numbers of plant OMTs chosen for sequence alignment in Figure 1 are as follows: MePi_4-FOMT from *Clarkia breweri* (AAD36946.1), OrSa_7-FOMT from *Oryza sativa* (BAM13734.1), Arth_3-FOMT from *Arabidopsis thaliana* (AA896879.1), ChAm_3-FOMT from *Chrysosplenium americanum* (AAA80579.1), MePi_3-FOMT from *Mentha piperita* (AA909601.1), OrSa_3-FOMT from *Oryza sativa* (XP_015650053.1), CaRo_4-FOMT from *Cicer arietinum* (XP_004489528.1), MeTr_7-IOMT from *Medicago truncatula* (AA185882.1), MeSa_7/4-FOMT from *Medicago sativa* (AAD49928.1). GiEc_4-IOMT from *Glycyrhiza echinata* (BAC58011.1), LoJa_4-FOMT from *Lotus japonicus* (BAC58013.1), MeTr_4-IOMT from *Medicago truncatula* (AA185881.1), CaRo_3-POMT from *Catharanthus roseus* (AAK021071.1), CBir_3-POMT from *Clarkia brevior* (O023760.1), LoPe_3-POMT from *Lolium perenne* (A012053.1), MeSa_3-POMT from *Medicago sativa* (AAB46623.1), CBir_4-POMT from *Clarkia brevior* (O04385.2).

### NMR and mass spectrometry analysis

NMR measurements were carried out on a Bruker Avance 600 MHz spectrometer equipped with an inverse \(1{\text{H}}/13{\text{C}}/15{\text{N}}/9{\text{F}}\) quadruple resonance cryoprobehead and z-field gradients. All compounds were dissolved in DMSO-\(d_6\), and \(\text{H}^1\) NMR, \(\text{C}^{13}\) NMR and DEPT-135 experiments were performed. Identification of single methylated products was done by comparing assignments against known standards. For the double-methylated products, additional 2D NMR experiments (NOESY, HSQC, HMBC) were performed. Data were processed and analyzed using TopSpin 4.0.7. All spectra were recorded at 25°C. All peaks were referenced towards the DMSO-\(d_6\) peak (\(\text{H}^1\): 2.50 ppm; \(\text{C}^{13}\): 39.51 ppm).

Mass spectrometry was performed using a Shimadzu LC-MS 8030 equipped with an ESI (electrospray ionization) source and a mass spectrometer, using the same column and mobile phase as in the HPLC analysis. Negative ionization mode was used and automatic MRM (multiple reaction monitoring) optimization was performed to acquire optimal fragmentation and maximal transmission of the desired product ions.

### Bioinformatic analysis

The bioinformatic analysis was performed with YASARA 19.7.20. First, the structure of 3REO was back-mutated to its wild-type sequence and the SAH was transformed to SAM by the addition of the methyl group and this structure was refined at pH 7.5, 25°C for 500 ps, taking a snapshot every 25 ps. The structure with the lowest energy was selected for further experiments. For the mutants, the respective amino acids were swapped with subsequent energy minimization. The same was performed for the preparation of the substrate molecules. The docking experiments were performed with VINA method, using the force field AMBER03 at 30°C. Five receptor molecules were prepared for each experiment and in each receptor 25 dockings were performed, and the resulting structures were clustered when RMSD was <5 Å. The catalytic active conformation with the higher binding energy was selected. Figures were prepared with PyMol.

### Accession numbers

The accession numbers of plant OMTs chosen for sequence alignment in Figure 1 are as follows: MePi_7-FOMT1 from *Mentha piperita* (AA905958.1), MeTr_7-FOMT7 from *Medicago truncatula* (ABD36946.1), OrSa_7-FOMT from *Oryza sativa* (BAM13734.1), Arth_3-FOMT from *Arabidopsis thaliana* (AA896879.1), ChAm_3-FOMT from *Chrysosplenium americanum* (AAA80579.1), MePi_3-FOMT from *Mentha piperita* (AA909601.1), OrSa_3-FOMT from *Oryza sativa* (XP_015650053.1), CaRo_4-FOMT from *Cicer arietinum* (XP_004489528.1), MeTr_7-IOMT from *Medicago truncatula* (AA185882.1), MeSa_7/4-FOMT from *Medicago sativa* (AAD49928.1). GiEc_4-IOMT from *Glycyrhiza echinata* (BAC58011.1), LoJa_4-FOMT from *Lotus japonicus* (BAC58013.1), MeTr_4-IOMT from *Medicago truncatula* (AA185881.1), CaRo_3-POMT from *Catharanthus roseus* (AAK021071.1), CBir_3-POMT from *Clarkia brevior* (O023760.1), LoPe_3-POMT from *Lolium perenne* (A012053.1), MeSa_3-POMT from *Medicago sativa* (AAB46623.1), CBir_4-POMT from *Clarkia brevior* (O04385.2).
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Conflict of Interest

The authors declare no conflict of interest.

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