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Tobacco *Ntomt2* Revisited: Multiple O-Methylation of Quercetin is Catalyzed by a Single Gene Product

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Abstract

Tobacco *NtOMT2* was previously reported as an inducible, o-diphenol O-methyltransferase (OMT) that exhibits similar preferences towards quercetin and caffeic acid as substrates. In this report, we further examine the substrate specificity of *NtOMT2* based on enzyme assays, kinetic data and phylogenetic analysis. We demonstrate that *NtOMT2* is a flavonol OMT that catalyzes multiple O-methylation of the flavonol quercetin giving rise to its mono-, di- and trimethyl ether derivatives. Mono-methylation of quercetin on the 3-, 3′-/4′- and 7-hydroxyl groups occurs in a random fashion according to the physio-chemical properties of their hydroxyl groups, together with their similar binding energies in the *NtOMT2* binding pocket. Homology modeling of *NtOMT2* with the *Medicago sativa* COMT as a template indicates that the H-bonding network between the functional hydroxyl groups and the neighboring residues allowed *NtOMT2* to establish a number of energetically similar binding arrangements with slightly different binding modes; thus resulting in a random multiple methylation. *NtOMT2* functions as a proximity and orientation agent in using the general base catalysis for multiple methylation of quercetin. This is the first report to indicate that quercetin multiple methylation is catalyzed by a single gene product.

Keywords: *Nicotiana tabacum* L.; Quercetin; O-Methylation; *NtOMT2*

Abbreviations: AdoMet: *S*-adenosyl-L-methionine; 5-HFA: 5-hydroxyferulic Acid; OMT: O-methyltransferase; ORF: Open Reading Frame; Q: Quercetin

Introduction

Flavonoid compounds constitute one of the largest groups of natural products that originate from the shikimate and polyketide pathways. They exhibit a wide range of functions and play important roles in the biochemistry, physiology, and ecology of plants. In addition, they are considered as an important part of the human diet and act as active principles for many medicinal plants.

Flavonoids are classified according to the oxidation level of the C-ring, and further enzymatic modifications such as methylation, glucosylation, sulfonation and (iso) prenylation that contribute to their diversity and biological activity. In plants, O-methylation of flavonoids is catalyzed by a family of **S**-adenosyl-L-methionine (AdoMet)-dependent O-methyltransferases (OMTs) [1]. Generally, O-methylation reduces the reactivity of hydroxyl groups, modifies their solubility and increases their anti-microbial and anti-cancer properties [2,3]. So far, more than 100 plant OMTs have been characterized or annotated in GenBank, of which the caffeic acid OMTs (COMTs) are most abundant [4].

The flavonol quercetin (3,5,7,3′,4′-pentahydroxyflavone; Figure 1) may be considered one of Nature's gifts to mankind, for its potential benefits to human health. It is of ubiquitous occurrence in plants and a common component of foods, especially fruits, vegetables and beverages such as tea, coffee, wine, beer and vinegar [5]. In addition, it is one of the most potent antioxidants derived from plant sources [6], and has been credited for its anti-cancer [7,8], anti-inflammatory [9], and anti-allergic [6,10] activities. Quercetin can be methylated to various methyl ether derivatives by plant OMTs, and several of these products were reported to occur naturally in the wild tobacco, *Nicotiana attenuata* [11]. Several flavonol- or quercetin-specific OMTs have been reported so far, based on enzyme essays of purified protein extracts, or the molecular cloning of their genes [12-18].

In tobacco (*Nicotiana tabacum* L. var. Samsun), three plant OMTs (NtOMT1, 2 and 3) have been reported [19]. NtOMT1 was mainly expressed in healthy tobacco leaves and shown to be involved in lignin biosynthesis; whereas *NtOMT2* and its isoform, *NtOMT3* were preferentially induced during the hypersensitive reaction to tobacco mosaic virus (TMV). However, *NtOMT2* exhibited similar substrate preferences towards quercetin and caffeic acid [19]. The *NtOMT2* gene was later cloned and characterized at the molecular level, and both *NtOMT2* and *NtOMT3* were considered to represent a new class

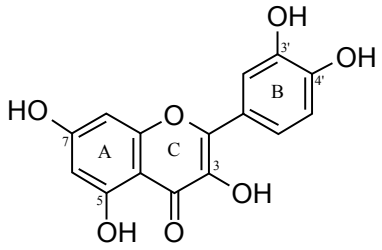

Figure 1: Structure of quercetin.

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of OMT genes associated with TMV infection, or treatment with the porteinaceous elicitor, megaspermin [20].

Multiple O-methylation of quercetin was first reported more than three decades ago by enzyme extracts of *Calamondin orange* peel and tobacco cell cultures, and methylation at the 3-position was later shown to be the first step in the enzymatic process [12,21,22]. This phenomenon was attributed, at the time, to the presence of several OMT species in the partially purified protein preparations. Furthermore, multiple glucosylation of quercetin has also been reported by the recombinant proteins of *Arabidopsis thaliana* [23] and *Medicago truncatulas* [24], although such phenomenon has not been further investigated.

In this paper, we further characterize *NtOMT2* enzyme activity, and examine the multiple methylation of quercetin. We report here that the multiple methylation of quercetin is catalzyed by a single gene product, and occurs in a random fashion according to the physico-chemical properties of its hydroxyl groups, together with their similar binding energies in the *NtOMT2* binding pocket.

Materials and Methods

Chemicals

Quercetin was purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario). *S*-Adenosyl-L-[^{3}H] methionine (80 Ci/mmol) was obtained from American Radio labeled Chemicals (St. Louis, MO). Most of the methylated derivatives used in this study were from our laboratory collection, or as generous gift from Professor Eckhard Wollenweber, Darmstadt, Germany. Unless otherwise specified, all other chemicals were of analytical grade.

Expression and purification of *NtOMT2* in *E. coli*

After sequence confirmation, the ORF of *NtOMT2* cDNA was amplified and subcloned into the expression vector pET200/D-TOPO (Invitrogen) for in vitro protein expression. The primers used for PCR were: *NtOMT2* F: 5'-CACCATGGAATCCTCAACCAAAAGCC; *NtOMT2* R: 5'-CTACTTGCAGAATTCCAT. PCR was conducted following the method described earlier [24]. The sequence and orientation of *NtOMT2* ORF in the expression vector were confirmed before chemically transformed into *E. coli* BL21 (DE3) cells (EMD, Darmstadt, Germany) for protein expression and purification as described before [24].

Enzyme assay and identification of reaction products

The standard enzyme assays were performed as previously described [24] using 50 µM of quercetin, its methylated derivatives or selected phenolic substrates, 50 µM AdoMet containing 25 nCi of the ^{3}H-label, and 0.1 to 2.0 µg of the affinity-purified recombinant protein. To study the influence of pH on *NtOMT2*, Bis-Tris buffer (pH 6.5), Tris-Cl buffer (pH 7.5) and Bicine buffer (pH 8.5) were used in the enzyme assays.

Characterization of the enzyme reaction products was carried out using TLC, HPLC and LC-MS, as well as comparisons with reference compounds. TLC and HPLC were performed as described earlier [25], except that the solvents used for HPLC were acetonitrile and 10 mM H_3PO_4. HPLC was performed in a linear gradient consisting of 50% of each solvent for 30 min. Identity of the reaction products was confirmed by comparing their retention times (R_t) and their UV absorption maxima with those of the reference compounds.

LC-MS analyses were carried using an Agilent 1200 HPLC system with a binary pump, in-line degasser, high performance auto-sampler and thermo-stated column division, using a linear gradient of MeOH-0.1% HCOOH in H_2O (40:60, v/v) for 30 min with a flow rate of 0.35 ml.min^{-1} on an Agilent SB-C18 column (2.1×30 mm; particle size, 3.5 µm), and a column temperature of 25°C. The system was optimized for UV detection at 254 nm and 340 nm as previously described [26]. Reference compounds were used to compare the corresponding retention times and mass spectral profiles of the methylated quercetin derivatives.

Kinetic analyses were performed using 10 µg of the affinity-purified *NtOMT2* proteins with a saturating concentration (100 µM) of AdoMet, containing 25 nCi of radioactivity, and varied concentrations (5 µM to 80 µM) of the substrates. The assays were performed in triplicates and were repeated twice. Lineweaver-Burk plots [27] were applied for the determination of K_m and V_{max} values.

Phylogenetic analysis

To study the phylogenetic relationship among *NtOMT2* and other plant OMTs, a number of closely related plant OMT amino acid sequences were aligned with *NtOMT2* using Clustal W [28]. Phylogenetic analysis was conducted using MEGA3 [29]. The neighbor-Joining method was employed to generate the tree, and bootstrap analysis (100 replicates) was applied to evaluate the reliability of the tree.

Homology Modeling and Molecular Docking

Homology modeling of *NtOMT2* was carried out using MsCOMT [30] as a template, as previously described [31]. Docking calculations were carried out using Docking Server (*http:// www.dockingserver.com*). The MMFF94 force field [32] was used for a preliminary energy minimization of the ligand molecule (quercetin) using Docking Server. PM3 semi empirical method was used in a second step to optimize the geometry of ligand molecule. PM3 semi empirical charges calculated by MOPAC2009 [33] were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on protein model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools [34]. Affinity (grid) maps of 20×20×20 Å grid points and 0.375 Å spacing were generated using the Auto grid program [34]. Auto Dock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method [35]. Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 2500000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied. Finally, molecular visualization and analysis were made using PyMOL 0.99 [36].

Site-directed mutagenesis

The mutants were prepared using Quick Change site-directed mutagenesis kit (Stratagene, CA), as described before [31]. The wild type *NtOMT2* cDNA in vector pET200/D-TOPO was used as a template for PCR. Primers used for generating mutants are shown in Table 1.

Results

NtOMT2 is a novel flavonol OMT

Three plant OMTs have been reported to occur in tobacco [19].

NtOMT1 is involved in lignin biosynthesis; whereas *NtOMT2* and its isoform, *NtOMT3* are induced by tobacco mosaic virus, indicating that they may have different functions. *NtOMT2* cDNA contains an ORF of 1098 bp that encodes a 365 amino acid polypeptide with a calculated molecular mass of 40.5 kDa and a theoretical isoelectric point of 5.27. Sequence analysis at the amino acid level showed that NtOMT1 and *NtOMT2* share 53% identity, and exhibit similar relative enzyme activities towards quercetin and caffeic acid [19].

In order to compare *NtOMT2* with NtOMT1 and other plant OMTs, we generated an amino acid sequence-based neighbor-joining tree. In this tree (Figure 2), *NtOMT2* appeared more phylogenetically distant from NtOMT1 and other COMTs, especially MsCOMT and PtCOMT which utilize both caffeic acid and 5-HFA as substrates, and are involved in lignin biosynthesis. This suggests that *NtOMT2* belongs to a different group of OMTs. It is also different from the wheat (*Triticum aestivum*) TaOMT2, a recently characterized flavone-specific OMT involved in tricin biosynthesis [25].

To re-examine the substrate preference of *NtOMT2*, we expressed its recombinant protein with a His-tag in vitro and affinity-purified it to near homogeneity (Figure 3). It was then assayed against a number of potential methyl acceptors, including caffeic acid, 5-HFA and a few flavonoid compounds, based on our phylogenetic analysis and previous reports. The results (Table 2) show that *NtOMT2* had the highest preference for quercetin as substrate, although it also utilized caffeic acid, luteolin (a flavone), myricetin (a flavonol) and 5HFA, albeit to a much lesser extent. It is interesting to note that, compared with quercetin, myricetin was a very poor methyl acceptor, possible because of the steric hindrance caused by an extra hydroxyl group on the B-ring.

To determine the best substrate for *NtOMT2*, kinetic analysis was performed using quercetin and caffeic acid as substrates. The results

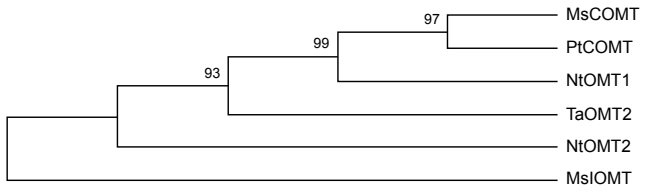

Figure 2: Neighbor-joining tree of NtOMT2 and other related OMTs at the protein level. Numbers at branching points are the bootstrap values indicating the confidence level for each branch based on 100 repeats. OMT sequences were obtained from GenBank with Accession numbers: aab46623, *Medicago sativa* MsCOMT; X62096, *Populus tremuloides* PtCOMT; ABB03907, *Triticum aestivum* TaOMT2; X74452, *Nicotiana tabacum* NtOMT1; X71430, *Nicotiana tabacum* NtOMT2; AAC49927, *Medicago sativa* isoflavone MsIOMT.

H271VF	GAAGTGGATTCTTgtgGACTGGAGTGATAG
H271VR	CTATCACTCCAGTCcacAAGAATCCACTTC
C318VF	GCGTTTCGCAAgtgGATTTGATCATG
C318VR	CATGATCAAATCcacTTGCGAAACGC
L131DF	GCCACTTTTGGCTgatCTTCAAGATAAAG
L131DR	CTTTATCTTGAAGatcAGCCAAAAGTGGC
L132DF	GGCCACTTTTGGCTTTGgaTCAAGATAAAGTATTC
L132DR	GAATACTTTATCTTGAtcCAAAGCCAAAAGTGGCC

* Mutated codons are underlined, with the lowercase letters indicating a base change from the wild-type sequence.

Table 1: Primers used for generating *NtOMT2* mutants*.

Substrate	Relative activity (%)	V_{max} (pkat.mg-1)	K_m (μM)	K_{cat}/K_m (mM-1.min-1)
Quercetin	100	500	128.5	1587.4
Caffeic acid	45.3	175.4	190.1	594.8
Luteolin	19.3			
Myricetin	3.6			
Tricetin	2.7			
5-HFA	2.1			

*The standard enzyme assay was used as described in the Methods section.

Table 2: Enzymatic activity of NtOMT2 with a number of selected substrates*.

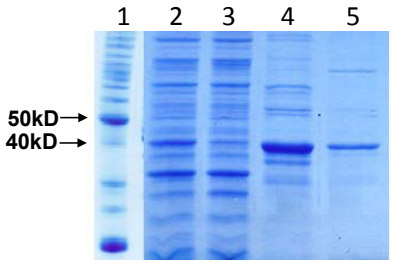

Figure 3: SDS-PAGE profiles of the recombinant NtOMT2 protein during affinity purification. 1. Mr Ladder of standard proteins; 2. *E.coli* lysate (10 μg); 3. wash; 4. Ni-eluate (1 μg); 5. desalted protein (0.2 μg).

indicated that quercetin was the preferred substrate for *NtOMT2* based on its K_m, V_{max} and $Kcat/_{Km}$ values (Table 2). The fact that 5HFA, a good lignin precursor, was the least effective methyl acceptor indicates that *NtOMT2* is not involved in lignin biosynthesis. Taken together, these results are in an agreement with our phylogenetic analysis and its physiogical role in the tobacco plant [19].

Characterization of quercetin reaction products

To examine the pattern of quercetin (Q) methylation products, [^{3}H]-labeled enzyme assays were analyzed by TLC and autoradiography (Figure 4), as well as HPLC of preparative scale, non-labeled assays (Figure 5). In a 10-min incubation, the products of Q methylation were characterized on TLC as 3-MeQ, 7-MeQ, 3′-MeQ, a diMeQ and a triMeQ (presumably 3,7,3′-triMeQ), albeit with different label intensities (Figure 4). The same pattern of methylated products was also observed on HPLC, except that 3-MeQ was the major compound. These products co-migrated on TLC and HPLC with authentic samples of Q mono-, di- and trimethyl ether derivatives. A comparison of the relative intensity of the [^{3}H]-label in 3-MeQ and tri-MeQ (Figure 4) and their amounts (Figure 5) seems to reflect their respective low and high specific activities, and suggests that Q methylation by *NtOMT2* proceeds towards the formation of trimethyl derivatives utilizing mono- and dimethyl intermediates, as is evident in a 60-min, steady-state incubation (Figure 4). In fact, when 3-MeQ was used as a substrate, 3,3′/3,4′-diMeQ was identified as the product, whereas 7-MeQ gave rise to 7,3′/7,4′-di-MeQ. However, no product was formed when 4′-MeQ was used as a substrate (data not shown). It should be noted that 3′-MeQ and 4′-MeQ, as well as 3,3′- and 3,4′-di-MeQ are difficult to separate, either on TLC or HPLC, mainly because of their similar physico-chemical properties [11].

Effect of pH on quercetin methylation

The fact that Q methylation profiles differed with changes in pH under standard enzyme assay prompted us to investigate the

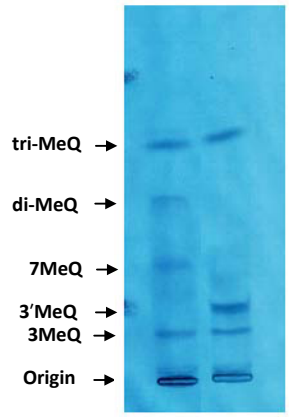

Figure 4: Autoradiogram of the chromatographed enzyme reaction products formed by the NtOMT2 recombinant protein with quercetin as the substrate after 10- and 60-min incubations. The TLC plate was exposed to Kodak BioMax MR film for 12 days.

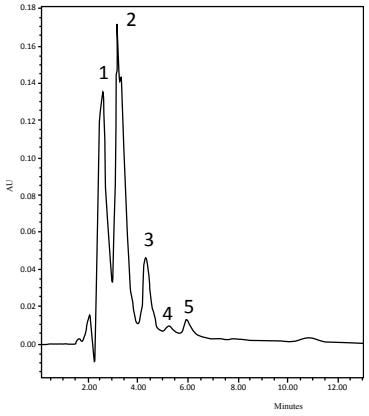

Figure 5: HPLC profile of a 10-min enzyme assay of NtOMT2 with quercetin (Q) as the substrate: 1. residual Q; 2. 3-MeQ; 3. 3′-MeQ; 4. 7-MeQ; 5. a di-MeQ.

phenomenon of multiple methylation in relation to the free energies of deprotonation/free energies of methylation of quercetin hydroxyl groups [37]. Therefore, *NtOMT2* enzyme assays were performed in three different pHs (6.5, 7.5 and 8.5) and the reaction products analyzed by LC-MS (Table 3). At low pH 6.5 the major product of methylation was identified as 3′-MeQ, together with a trace amount of 7-MeQ. When the pH was increased to 7.5, the pattern of methylation changed to 3-MeQ> 3,3′-di-MeQ >>7,3′-di-MeQ. At pH 8.5, the major product of methylation was 3′-MeQ, with a trace amount of 7,3′-di-Me derivative (Table 3). These results are in agreement with the reported O–H bond dissociation enthalpy (BDE) where 3-OH> 3′-OH> 7-OH [37]. Therefore, under physiological conditions (pH 7.5) the 3- and 3′-hydroxyls are the more preferred sites of methylation, whereas at lower or higher pHs only the 3′- and 7-hydroxyls are methylated, with the latter being much less favored.

Substrate binding and catalysis

The fact that *NtOMT2* catalyzed the multiple methylation of quercetin, prompted us to further investigate this phenomenon at the structural level. *NtOMT2*, which exhibits 56.1% identity to the crystallized MsCOMT (PDB, 1KYZ) [30], was used in a homology modeling approach to study its structural basis for substrate binding and catalysis. After modeling, quercetin and AdoMet were docked into the substrate binding pocket (Figure 7) using Docking Server. Superimposition of the modeled *NtOMT2* with that of MsCOMT revealed that most of the highly conserved residues of *NtOMT2* are located in the C-terminal portion of the protein that includes the signatures of most plant Class II OMTs [38,39].

NtOMT2 transfers the methyl group of AdoMet to the hydroxyl groups of quercetin, giving rise to various methylated derivatives and AdoHcy as products. In *NtOMT2*, AdoMet is bound in a similar manner as in MsCOMT (Table 4). Based on docking results, several H-bonds were observed extending from the ligands and the functional groups of their neighboring residues. The H-bonding network allowed *NtOMT2* to establish a number of energetically similar binding arrangements with slightly different binding modes.

In order to determine the critical residues involved in the phenolic substrate binding, different residues such as Leu131, Leu132 and Cys318 were chosen for site-directed mutagenesis based on comparison of the residues surrounding the binding site (Table 5), and the mutant proteins analyzed for enzyme activity. Only mutant protein C318V showed difference with *NtOMT2* wild protein when assayed with Q. The HPLC (Figure 6) shows that such mutation resulted in the loss of multiple O-methylation, and the formation of only one product, 3-MeQ, albeit in a small amount. This mutation experiment confirmed the crucial role of Cys318 in the sequential methylation of quercetin by *NtOMT2*, and conferred credibility to the homology model and docking procedure used.

In most structurally characterized MTs, the transmethylation reaction was reported to be mediated by a general acid-base catalysis using a histidine residue [30,31,40]. In the case of *NtOMT2*, we propose His271 (equivalent to His269 in MsCOMT), as the deprotonating residue of the substrate (Figure 7). Mutation of His271 to Val completely abolished the catalytic activity of the mutant protein (data not shown), indicating the importance of this residue in methylation. As was with other small-molecule OMTs, methyl transfer in *NtOMT2* is proposed to be catalyzed by a nucleophilic attack of the resulting phenolate anion on the reactive methyl group of AdoMet. It is proposed that the methylation sequence proceeds through proximity and orientation effects [41], as

pH	Reaction products
6.5	3'-MeQ>> 7-MeQ>> 7,3'-diMeQ
7.5	3-MeQ> 3,3′/3,4'-diMeQ>> 7,3′/7,4'-diMeQ
8.5	3'-MeQ> 3,7-diMeQ

*Enzyme assays were conducted as described in the Methods section.
Table 3: Reaction products of NtOMT2 enzyme assays with quercetin as substrate under different pH's*.

OMT	Residues							
MsCOMT	Asp206	Gly208	Asp231	Leu232	Asp251	Met252	Lys265	Trp271
NtOMT2	Asp208	Gly210	Asp233	Leu234	Asp253	Met254	Lys267	Trp273

Table 4: Residues in NtOMT2 and MsCOMT neighbouring the AdoMet binding site.

OMT	Residues							
MsCOMT	Met130	Asn131	Phe176	Met180	Ile316	Ile319	Met320	Asn324
NtOMT2	Leu131	Leu132	Phe178	Met182	Cys318	Ile321	Met322	Asn326

Table 5: Residues in NtOMT2 and MsCOMT neighbouring the phenolic substrate binding site.

well as changes in the hydrogen bonding environment created by the amino acid residues neighboring the different ligands. This would also allow the sequential methyl transfers to take place without the need for transfer of the methylated intermediate to another catalytic site.

Docking experiments indicated that there was no strong, energetically-favorable position for all *NtOMT2*-quercetin conformers (Table 6). This assertion is supported by the observed *NtOMT2* enzyme activity with quercetin as the substrate (Figure 4 and 5). The sequence of multiple mono-methylation of quercetin is not critical. It is assumed that the methyl transfer reactions can begin at any OH group of quercetin at random, followed by further methylation to give rise to various di- and trimethyl derivatives.

Discussions

Multiple O-methylation of quercetin has been reported to be catalyzed by individual OMTs [12,16-18]. We have demonstrated that *NtOMT2*, a single gene product, can catalyze the methylation of different hydroxyl groups of quercetin. *NtOMT2* is a novel flavonol OMT. It is different from other reported flavonol OMTs, such as Arabidopsis AtOMT1 which catalyzes a single methylation of quercetin

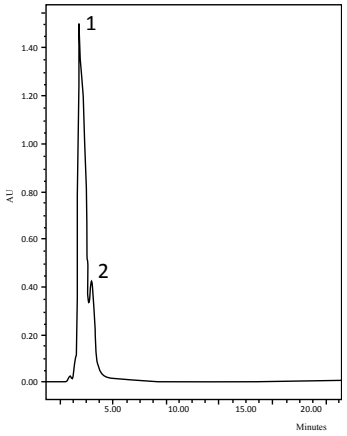

Figure 6: HPLC profile of the mutant protein V318 reaction product with quercetin (Q) as the substrate: 1. residual Q; 2. 3-MeQ.

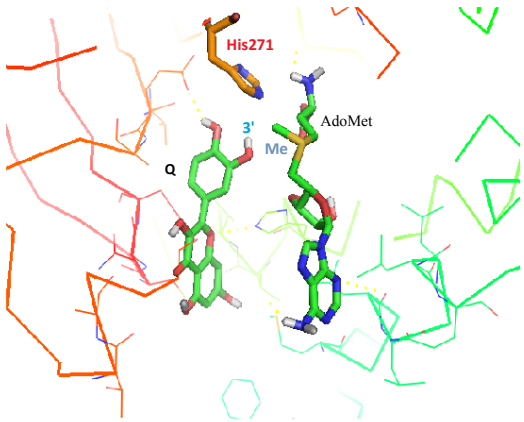

Figure 7: Docking image showing the catalytic residue (His271), substrate quercetin (Q) and AdoMet. The methylated position of quercetin (3′) and the methyl group (Me) of doner AdoMet are indicated.

Ligand	Adjacent Products from assay* -OH	Distance to His271(A°)	Distance to AdoMet-S(A°)	H-bond	vdW+Hbond+ desolvation energy
Quercetin 3'-MeQ	3'-OH	2.6	4	His185•••1O	178.82nM
3-MeQ	3-OH	4.6	6.6	Asp272•••4'OH	3-MeQ
				Glu299•••4'OH	
7-MeQ	7-OH	5.1	5.9	Asp272···3'OH	
				Leu128···7OH	
				Gln325···5OH	146.01nM
				Cyc318···7OH	
3-MeQ	3'-OH	3.9	11.2	Asp272···3'OH	N/A
3, 3'-diMeQ	4'-OH	3.8	10.8	Asp272···4'OH	
				Leu128···7OH	
3'-MeQ	4'-OH	3.7	7	Glu299···4'OH	N/A
3',4'-diMeQ				His185···1O	
7-MeQ	3'-OH	4.2	5.3	His185···1O	N/A
7, 3'-diMeQ	4'-OH	4.6	6.6	Glu331···3'OH	

*Reaction products were characterized by TLC, HPLC and LC-MS as described in the Methods section.

Table 6: NtOMT2 modeling data and reaction products from enzyme assays.

to its 3′-methyl derivative [15] or the tomato, trichome-specific OMTs which methylate myricetin at its 3′/5′- or 7/4′-positions [18]. NaOMT2 is quercetin-specific and methylates its 3-, 7- and 3′-hydroxyl groups, albeit with various degrees. The mono-methylated products are further methylated to di- and trimethylated products. While this phenomenon seems to occur in a random fashion, it is dependent on the environmental conditions, especially the pH of the assay buffer. This suggests that the mechanism involved in the multiple methylation is different from the sequential-type methylation that is catalyzed by the wheat flavone TaOMT2. In the latter case, tricetin (5,7,3',4',5'-pentahydroxyflavone) is methylated to its 3'-methyl- (selgin), 3',5'-dimethyl- (tricin) and 3',4',5'-trimethyl ether derivatives [23]. The acidity and activity of the different hydroxyl groups are dependent on the polarity of the solvent. In polar solvents, position 7 is the most acidic site, followed by position 3′/4′ and 3. This is consistent with the experimental pKa values of 7.7, 8.8 and 9.8 obtained for the three most acidic groups: 7-OH, 4′-OH and 3-OH respectively [42]. On the other hand, at low pH, none of these positions is deprotonated, so that the easiest site to be methylated is clearly the 3′/4′-OH, followed by the 7-OH, 3-OH groups and some di-methylation. When the pH increases, deprotonation occurs on three different sites, which allows the mono-methylated products to be further methylated to di- and even tri-methyl quercetin derivatives, as shown in Figure 4. It was also shown that the extent of flavonoid methylation correlated well with the negative electron densities of the different hydroxyl groups of quercetin as well as other hydroxyflavones [12], as calculated by the complete neglect of differential overlap (CNDO/2) theory [43].

The homology modeling approach used in this study demonstrated that the similar binding energies of the different OH groups in the *NtOMT2* binding pocket (Table 6) seem to be responsible for the random fashion of mono-methylation, which is in agreement with our experimental data. Furthermore, the low level of mono-methylation and multiple methylations may be explained by a weak binding affinity of the enzyme.

Finally, methylated flavones have been reported to be most effective compounds for cancer prevention [44]. Among the various quercetin methylated derivatives, 3-MeQ has been shown to have many potential benefits for human beings. In this study, we demonstrated that Cys318 is the most critical residue involved in the multiple O-methylation of quercetin. It was possible to replace this amino acid with a different residue in favour of 3-MeQ production. We are pursuing this line of research with the view to increase the yield of this valuable metabolite.

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