Structural Modeling and Biochemical Characterization of Flavonoid *O*-Methyltransferase from Rice

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Natural products from plants are diverse, and might be used as novel starting materials for pharmatheutical or neutrotheutical purposes. Plant phenylpropanoids, such as lignins, flavonoids, and anthocyanins, are a group containing abundant natural compounds that exert diverse biological effects on humans, as well as plants.[1] Phenylpropanoids derived from the amino acid phenylalanine by phenylalanine ammonia lyase (PAL) are synthesized *via* the phenylpropanoid pathway.[2] The hydroxyl groups of phenolic compounds are chemically active, and thus undergo several modification reactions, which result not only in structural diversity but in alterations in biological activities.[3] *O*-Methylation and *O*-glycosylation are common modification reactions.[4,5] The *O*-methyltransferases (OMTs) mediate a transfer reaction in which hydroxyl groups of phenolic compounds serve as methyl group acceptors, and *S*-adenosylmethionine (SAM) functions as a donor. *O*-Methylation induces a reduction in the chemical reactivity of phenolic hydroxyl groups, and an increase in antimicrobial activity.[6]

OMTs that utilize phenylpropanoids are represented by two groups: caffeoyl coenzyme A OMTs (CCoAOMTs) and caffeic acid OMTs (COMTs).[7] Originally, CCoAOMT and COMT played roles in lignin biosynthesis. Eventually, other substrates were found that utilized both groups of OMTs. The amino acid sequences and substrate ranges of these two OMTs differ.[8] Moreover, CCoAOMTs have a molecular weight ranging from 23,000 to 27,000 and are Mg^{2+} dependent, whereas the family of COMTs have molecular weights of approximately 38,000 to 43,000, and do not require Mg^{2+} for their catalytic activity. The OMTs that utilize flavonoids and alkaloids are more similar to COMTs. Therefore, it has been generally accepted that the OMTs that are similar to CCoAOMTs have a substrate range distinct from those that are similar to COMT. However, recent studies have demonstrated that OMTs closely related to CCoAOMT from microorganisms and plants utilized flavonoids more efficiently than caffeoryl-CoA.[9-12] However, it remains unclear as to whether these OMTs constitute a new group of OMTs. The structures of OMTs of both groups have been determined and the reaction mechanisms of OMTs have been elucidated.[13-15]

Previously, we cloned and characterized flavonoid OMT from rice (ROMT9).[16] ROMT9 is a B-ring-specific flavonoid OMT. When tricetin is utilized, ROMT-9 immediately transfers two methyl groups to the 3' and 5'-hydroxyl groups of

tricetin and forms tricin (3',5'-*O*-dimethyltricetin) (Fig. 1). The 3'-*O*-methyltricetin (selgin) (Fig. 1) was rarely observed even after a longer enzyme reaction period (Fig. 3A). In order to evaluate the molecular basis of this phenomenon, the structure of ROMT-9 was reconstructed using molecular modeling techniques, with the caffeic acid/5-hydroxyferulic acid 3/5 *O*-methyltransferase (COMT) from *Medicago truncatula* (PDB ID: 1kyz) being used as a template. The overall structure of ROMT9 is similar to that of the COMT from *M. truncatula*.[15] Like COMT, the C-terminal domains of ROMT9 are principally involved in binding to substrates (flavonoid and *S*-adenosylmethionine (SAM)). The flavonoid binding site forms a narrow channel, which was also noted in COMT (Fig. 2). The SAM binding site of ROMT9 is more spacious than the flavonoid binding site. This narrow channel appears to be important for the positioning of flavonoids, as no specific interaction between tricetin and ROMT9 was observed, with the exception of a few hydrogen bonds (see below).

Figure 1. Structure of flavonoids used in this study and reaction scheme of ROMT-9 with tricetin. A, B, and C indicate A, B and C rings of flavonoids.
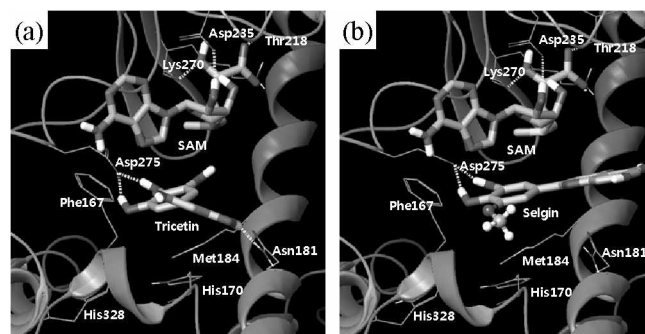

Figure 2. Docking structure of tricetin (a) and selgin (b) into the active site of ROMT9. Amino acid residues interacting with either flavonoids or SAM are indicated.

In order to assess the binding of either tricetin or selgin to the substrate binding sites, both tricetin and selgin along with SAM were individually docked into the substrate binding site of ROMT9. As mentioned above, the flavonoid binding sites of tricetin and selgin formed a long channel. The B ring of tricetin (Fig. 2A) and selgin (Fig. 2B) was located on the inside of this channel, which brings the 3' or 5' hydroxyl group of flavonoids close to the methyl donor, SAM. The hydrogen bonds between Asp275 and the 3' and 4'-hydroxyl groups of tricetin or the 4' and 5'-hydroxyl groups of selgin were noted (Fig. 2). Additionally, Asn181 forms a hydrogen bond only with the carbonyl group of tricetin (Fig. 2A). The docking configurations of tricetin and selgin differed due to the 3'-methoxy group of selgin. When the B rings of the two flavonoids overlap, the A and C rings were rotated approximately 90° relative to each other (Fig. 2). The 3'-methoxy group of selgin is located within a hydrophobic pocket formed by Phe167, His328, His170, Asn181, and Met184 (Fig. 2B). Among the amino acids that participate in the formation of the hydrophobic pocket, His328 was predicted to perform a crucial role in the formation of the pocket.

The SAM binding site of ROMT9 forms three hydrogen bonds: the methionine part of SAM forms hydrogen bonds with Thr218 and Lys270, while the ribose part of SAM forms hydrogen bonds with Asp235 (Fig. 2). The SAM binding sites of various OMTs are conserved. And, the hydrogen bonds formed between SAM and OMTs guide the methyl group of SAM near to the methyl acceptor group of flavonoids for transmethylation.[3,14-15] Flavonoids bind into the narrow channel, while SAM, which is held by several hydrogen bonds, may create a more spacious region, which is located near the channel. This configuration would provide substrate selectivity in ROMT.

In an effort to determine the roles of amino acids as predicted by molecular docking, site-directed mutagenesis was conducted. From the modeled structure of ROMT9, Asn275 formed a hydrogen bond with a substrate, either tricein or selgin. The abolition of this hydrogen bond would result in the loss of enzymatic activity. Asn275 was mutated into Leu. The resulting Asn275Leu mutant was expressed in *E. coli* and purified. The mutant was purified identically to the wild-type. The reaction of the Asn275Leu mutant with tricetin demonstrated that the mutant retained only 3% of the activity of the wild-type strain. This suggested that the hydrogen bond between Asn275 and the substrate is important for the reaction, as was predicted. However, this mutation did not provide a rationale as to why ROMT9 generated tricin from tricetin without the formation of selgin. In fact, the HPLC profile of the product of the reaction of Asn275Leu with tricetin was identical to that of the wild-type (Fig. 3A, 3B). Asn181, which formed a hydrogen bond only with tricetin but not with selgin, was mutated to leucin. The resulting mutant, Asn181Leu, retained only 20% activity of the wild type (Fig. 3D). However, Asn181Leu showed the identical reaction product to the wild type, which indicates that this mutant did not change its substrate specificity and regioselectivity. These results showed that the hydrogen bonds predicted by the docking experiment play a crucial role in the reaction.
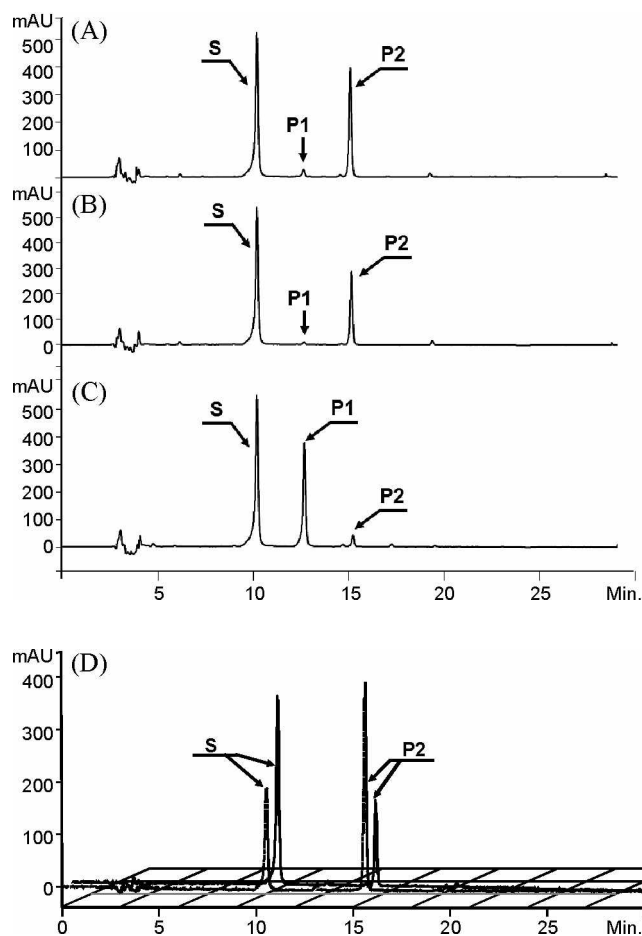

Figure 3. Analysis of reaction product from wild-type ROMT9 (A), Asn275Leu (B), His328Arg (C) with tricetin using HPLC. Reaction condition is described in experimental section. (D) Analysis of reaction product from N181L (a solid line) or ROMT9 (a dotted line) with tricetin. 40 μM of tricetin and 5 μg of the purified protein were used. S, tricetin; P1, selgin; P2, tricin.

His328 was located at the center of a pocket into which the 3'-*O*-methyl group of selgin fitted. The hydrophobic pocket formed by Phe167, His328, His170, Asn181, and Met184 is likely to endow selectivity for selgin. Thus, the destruction of this hydrophobic pocket would cause the selgin not to fit into the substrate binding site, or to be released from it. Thus, it was predicted that the mono methylated reaction (selgin) of the tricetin product would be observed. His328 was mutated into Arg in order to manipulate the hydrophobic pocket. The His328Arg mutant was expressed and purified. The analysis of the reaction product with tricetin revealed the mono methylate product, selgin (Fig. 3C). Thus, the hydrophobic pocket is critical for the positioning of selgin for the second methylation.

It remains to be determined how the selgin was methylated into tricin immediately after it was formed. It appears that selgin undergoes the second methylation reaction without leaving the substrate binding site, as the positioning of the flavonoid through the narrow channel to the flavonoid binding site of ROMT9 would be prohibitively time-demanding. According to the docking results, selgin must be rotated in the flavonoid binding site of ROMT9. It would be intriguing to

know how flexible the active site is for the rotation of selgin for the second methylation reaction. On the other hand. SAM is likely to be effectively supplied for the second methylation. The SAM binding site is more exposed than the flavonoid binding site. Thus, the entrance of SAM is substantially easier, and thus SAM may not be the limiting factor in the reaction.

Experimental Sections

Modeling of ROMT9. The sequence of ROMT9 (GenBank Accession No. 29893141) was submitted to the SWISS-MODEL (Automated Protein Modeling Server) database. The caffeic acid/5-hydroxyferulic acid 3/5 O-methyltransferase (COMT) from *Medicago truncatula* (PDB ID: 1kyz) was selected owing to its 60% sequence identity. The homology modeling of ROMT9 using COMT from *M. truncatula* was conducted using the homology modeling software Prime, incorporated into the Schrodinger modeling software suite. The optimal model was selected on the basis of the bond angle stereochemistry using PROCHECK (Laskowski *et al.* 1993).[17] Followed by refinement of the loop structures, the homology model was subjected to 10,000 steps of energy minimization using MacroModel software (www.schrodinger.com). Tricetin and selgin, as well as SAM, were docked into the active site of ROMT9 to determine the binding mode of these compounds. The default setting of the extreme precision mode of GLIDE (www.schrodinger.com) was employed for the docking, and up to 10 poses were saved for analysis. All of the saved poses were similar, and thus the top-scored pose was selected for the binding mode analysis.

Site-directed mutagenesis and enzyme assay. Site-directed mutagenesis was conducted using a QuikChange II XL site-directed mutagenesis kit (Stratagene. La Jolla, USA). The primers utilized for mutagenesis are listed in Table 1. The recombination proteins were expressed and purified via the method developed by Kim *et al.*[18] The O-methyltransferase assay was conducted as previously described.[11] Reaction mixture contained 60 μM of flavonoid, 500 μM of SAM and 3 μg of the purified wild type ROMT9 or 90 μg of D275L or 65 μg of H328R in 500 μL of 50 mM Tris/HCl (pH 7.5). The reaction mixture was incubated at 37 °C for 30 min. The organic layer was evaporated to dryness and the resulting samples were dissolved in metanol. Flavonoids were also analyzed by high performance liquid chromatography.[11]

Table 1. Lists of primers for the site directed mutagenesis of ROMT9.

Mutant	Primer
Asp275Leu	AAGTGGATCCTCCACCtcTGGAGCGACGAGCAC
His328Arg	ATGCTCGCCCgCAACCCCGGC
Asn181Leu	TCAACCGCGTCTTCctaGAGGGCATGAAGAACC

Position of mutation was indicated as small letters.

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