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ORIGINAL RESEARCH

Characterization of Phenylpropanoid *O*-Methyltransferase from Rice: Molecular Basis for the Different Reactivity Toward Different Substrates

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Abstract *O*-Methyltransferases (OMTs) transfer a methyl group from *S*-adenosylmethionine to a hydroxyl group of an acceptor. One group of OMTs is the caffeoyl-CoA *O*-methyltransferase type, which is involved in the biosynthesis of monolignol. In this study, *OsOMT26* was cloned from *Oryza sativa* and the recombinant OsOMT26 protein was characterized. OsOMT26 used not only caffeoyl-CoA as a substrate but also different flavonoids such as luteolin and tricetin. However, when caffeoyl-CoA was used as the substrate, the reactivity of OsOMT26 was approximately 6.6-fold better than when either luteolin or tricetin was used. This result demonstrated that OsOMT26 displayed the typical properties characteristic of CCoAOMT. Molecular modeling followed by site-directed mutagenesis was employed to examine why caffeic acid or caffeoyl-CoA was a better substrate than tricetin. One amino acid, Asp210, turned out to be critical for substrate binding, and site-directed mutagenesis of Asp to Glu improved the enzyme's reactivity toward tricetin.

Keywords *O*-Methyltransferase · Molecular modeling · *Oryza sativa* · Phenylpropanoid

Abbreviations

CCoAOMT	Caffeoyl-CoA OMT
COMTs	Caffeic acid OMTs
4-CL	4-Coumaroyl-CoA ligase
OMT	*O*-Methyltransferase
SAM	*S*-Adenosylmethionine

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Introduction

Methylation is a ubiquitous reaction found not only in animals but also in bacteria and plants. Depending on the methyl group acceptor, methylation can be divided into *C*-methylation, *N*-methylation, *O*-methylation, and *S*-methylation (Schubert et al. 2003). Research on methylations in animals and bacteria mainly has been focused on DNA or RNA, which are mediated by *N*-methyltransferases (Bügl et al. 2002; Bheemanaik et al. 2006). Although DNA or RNA methylation has also been studied in plants, *O*-methylation of secondary metabolites from plants is prevalent. *O*-Methylation of the secondary metabolites reduces the reactivity of hydroxyl groups, increases their hydrophobicity, and modifies their intracellular compartmentation (Ibrahim et al. 1987).

O-Methyltransferases (OMTs) catalyzes the transfer of a methyl group from *S*-adenosylmethionine (SAM) to the hydroxyl group of a methyl acceptor. In plants, many OMTs involved in the biosynthesis of secondary metabolites have been characterized (Lam et al. 2007). OMTs are classified based on their substrates and amino acid sequence. OMTs from plants are usually divided into two groups: caffeic acid OMTs (COMTs) and caffeoyl-CoA OMTs (CCoAOMTs). This classification is based on their substrate and the molecular weight of the OMTs. The molecular weight of COMTs, which ranges about 38–43 kDa, is approximately 15–16 kDa larger than that of CCoAOMTs. The substrate preference of the two classes of OMTs is different: CCoAOMTs prefer caffoyl-CoA and mainly participate in monolignol, while COMTs use other phenylpropanoids including flavonoids and alkaloids (Ferrer et al. 2005; Joshi and Chiang 1998).

O-Methylation, along with hydroxylation and reduction, leads to the biosynthesis of coniferyl and sinapyl alcohols from *p*-coumaryl alcohol, which is a monolignol (Weng et al. 2008). These monolignols eventually become *p*-hydroxylphenyl (H), guaiacyl (G), and syringyl (S) units of a lignin polymer, respectively (Rastogi and Dwivedi 2008). During this process, COMT transfers a methyl group to phenylpropanoids and CCoAOMT does the coenzyme A esters of the phenylpropanoids (Whetten and Sederoff 1995). Thus, two distinct classes of OMTs take part in these processes.

In this report, an OMT from rice was characterized. In addition, we examined why this OMT displayed better activity toward caffeic acid or caffeoyl-CoA than flavones such as luteolin or tricein using molecular modeling and site-directed mutagenesis.

Materials and Methods

Chemicals

Caffeic acid was purchased from Sigma (St. Louis, MO, USA) and flavonoids were obtained from Indofinechemicals (Hillsborough, NJ, USA). Caffeoyl-CoA was synthesized in our lab using 4-Coumaroyl-CoA ligase (4-CL) from rice (Lee et al. 2007).

Cloning and Expression of *OsOMT26*

Total RNA from the fresh leaves and stems of *Oryza sativa* Dongjin grown for 4 weeks was isolated using a Qiagen plant total RNA isolation kit (Qiagen, Hilden, Germany). cDNA was synthesized with 2 μg of total RNA using Omniscript reverse transcriptase (Qiagen). Full-length cDNA was cloned by PCR with the following primers: 5′-ATCGCCATGACGACCGGC-3′ and 5′-TGGATCGGCCGG TAAGGTCT-3′ (GenBank accession no. AB110168.1). The PCR products were subcloned into a pGEM-T easy vector (Promega, Madison, WI, USA) and the resulting plasmids sequenced. The nucleotide sequences of both OMTs match exactly the published sequence.

The open reading frame of *OsOMT26* was subcloned into an *Escherichia coli* expression vector pGEX-5X-3 (Amersham Biotech, USA) for the production of recombinant proteins. The expression and purification of the recombinant proteins were carried out as described in Kim et al. (2006a, b). Site-directed mutagenesis was carried out using a QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The primers for the Asp210Glu mutation were 5′-CTGTCGGACCTGgaaCG-GAGGTTCTCCG-3′ and 5′-CGGAGAACCTCCGttc-CAGGTCCGACAG-3′ (nucleotides for mutagenesis were indicated as lowercase letters). The mutation was verified by sequencing.

Enzymatic Reaction and Analysis of Reaction Products

The reaction mixture contained 0.25–1.25 μg of purified recombinant protein, 200 μM SAM, and 80 μM substrate in 50 mM Tris/HCl buffer (pH 8.0) in a final volume of 200 μl. The reaction was carried out at 37°C for 30 min. The reaction was stopped by adding 10 μl of 5 N HCl. The reaction mixture was extracted twice with an equal volume of ethyl acetate. The ethyl acetate layer containing the substrate and reaction product was dried completely under vacuum.

Reaction products were analyzed using a Varian high-performance liquid chromatography equipped with a photo diode array detector and a Varian C18 reversed-phase column (4.60×250 mm, 3.5-μm particle size; Varian, Palo Alto, CA, USA). The mobile phase consisted of 0.1% formic acid buffer (pH 3.0). The time-dependent gradient used for HPLC was 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 290 and 340 nm.

Analysis of samples using mass spectrometer was carried out as described (Kim et al. 2008, 2009).

Molecular Modeling of OsOMT26

The three-dimensional structure of OsOMT26 was modeled using the crystallographic structure of CCoAOMT (PDB ID: 1SUI) obtained from the Protein Data Bank. PRIME software in the Schrodinger modeling software suite was used for homology modeling. The three-dimensional model structure of OsOMT26 was then analyzed by the PROCHECK software. The model structure was subjected to conjugate gradient (PRCG) energy minimization with the OPLS-AA force field using the implicit GB/SA water model. The energy-minimized structure was further relaxed by molecular dynamics simulation (MD), which was performed by pre-equilibration of the model structure for 100 ps followed by simulation for 1 ns at 300 K with a 1-fs time step. During the MD simulation, the SHAKE algorithm was applied to all hydrogen bonds. The model structure of OsOMT26 obtained after MD simulation was chosen for docking the ligand, tricetin. GLIDE software (http://www.schrodinger.com) was used for the docking study. The default setting of the extreme precision mode of GLIDE was employed for docking, and among the top ten poses that were saved, the best scored pose was chosen for binding mode analysis.

Results and Discussion

Molecular Cloning and Characterization of *OsOMT26*

The open reading frame (ORF) of *OsOMT26* is 759 bp encoding a 27.8-kDa protein. OsOMT26 contains a conserved SAM-binding site as well as a metal-binding site (Asp159, Asp190, and Asn191). As mentioned, OMTs can be divided into two classes: class I represented by COMT and class II represented by CCoAOMT. The phylogenetic analysis of several OMTs also supports this classification. The class II OMTs can be divided into two groups: the CCoAOMT type and the CCoAOMT-like type. OsOMT26 belongs to the CCoAOMT (Fig. 1).

OsOMT26 was expressed as a glutathione *S*-transferase fusion protein in *E. coli* and purified to near homogeneity (data not shown). Several phenolic compounds such as caffeoyl-CoA and caffeic acid were tested as potential substrates for OsOMT26. Caffeoyl-CoA and caffeic acid were found to be appropriate substrates for OsOMT26. Mass spectrometry analysis of the reaction products revealed that the molecular mass of each reaction product increased by 14 Da compared with the substrate, indicating that one methyl group was attached. In addition, the HPLC retention

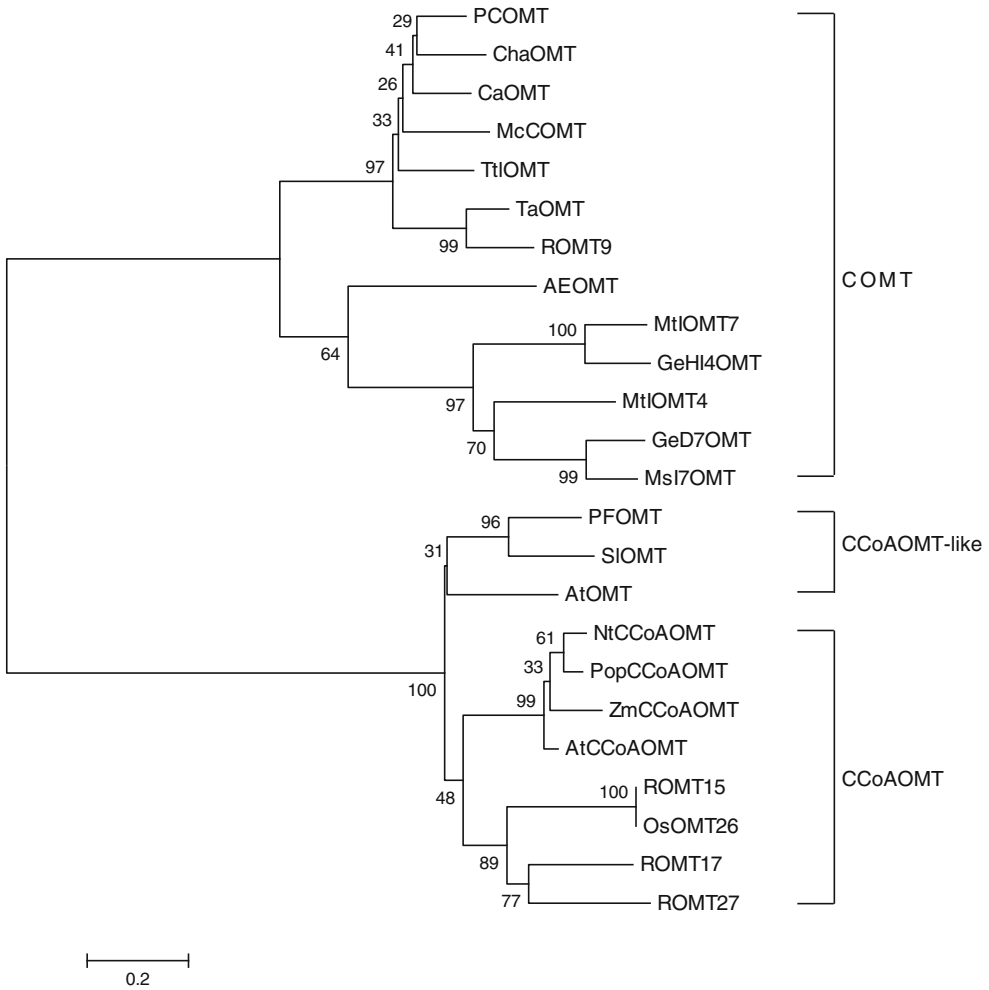

Fig. 1 Phylogenetic analysis of OMTs from various plants. PCOMT caffeic acid 3-OMT from *Populus deltoids* (EU078958.1), ChaOMT caffeic acid OMT from *Chrysosplenium americanum* (U16793.1), CaOMT caffeic acid OMT from *Capsicum annuum* (AF212316.1), McCOMT caffeic acid 3-OMT from *Mesembryanthemum crystallinum* (AF067968.1), TtlOMT catechol OMT from *Thalictrum tuberosum* (AF064696), TaOMT flavonoid OMT from *Triticum aestivum* (DQ223971.1), ROMT9 flavonoid OMT from *O. sativa* (DQ288259.1), AEOMT caffeic acid OMT from *Pinus taeda* (U39301.1), MtIOMT7 isoflavonoid OMT7 from *Medicago truncatula* (DQ419912.1), GeD7OMT daidzein OMT from *Glycyrrhiza echinata* (AB091685.1), Msl7OMT isoflavone 7-OMT from *Medicago sativa* (U97125.1), PFOMT OMT from *M. crystallinum* (AY145521.1), SlOMT caffeoyl-CoA 3-OMT from *Stellaria longipes* (L22203.1), AtOMT caffeoyl-CoA 3-OMT from *Arabidopsis thaliana* (NM_118755.3), NtCCoAOMT caffeoyl-CoA 3-OMT from *Nicotiana tabacum* (AF022775.1), PopCCoAOMT caffeoyl-CoA 3-OMT from *Populus trichocarpa* (XM_002313089.1), ZmCCoAOMT caffeoyl-CoA 3-OMT from *Zea mays* (EU952463.1), ROMT15 caffeoyl-CoA 3-OMT from *O. sativa* (AB110168.1), OsOMT26 OMT from *O. sativa* (AB110168.1), ROMT17 caffeoyl-CoA 3-OMT from *O. sativa* (NM_001068678), ROMT27 caffeoyl-CoA 3-OMT from *O. sativa* (NM_001068679.1)

time of the reaction products from caffeoyl-CoA and caffeic acid was indistinguishable from that of feruoyl-CoA and ferulic acid, respectively (data not shown). These results indicated that OsOMT26 used not only caffeoyl-CoA but also caffeic acid as a substrate. In addition, several flavonoids including apigenin, narigenin, kaempferol, luteolin, quercetin, and tricetin were tested. Among these flavonoids, only luteolin, quercetin, and tricetin resulted in

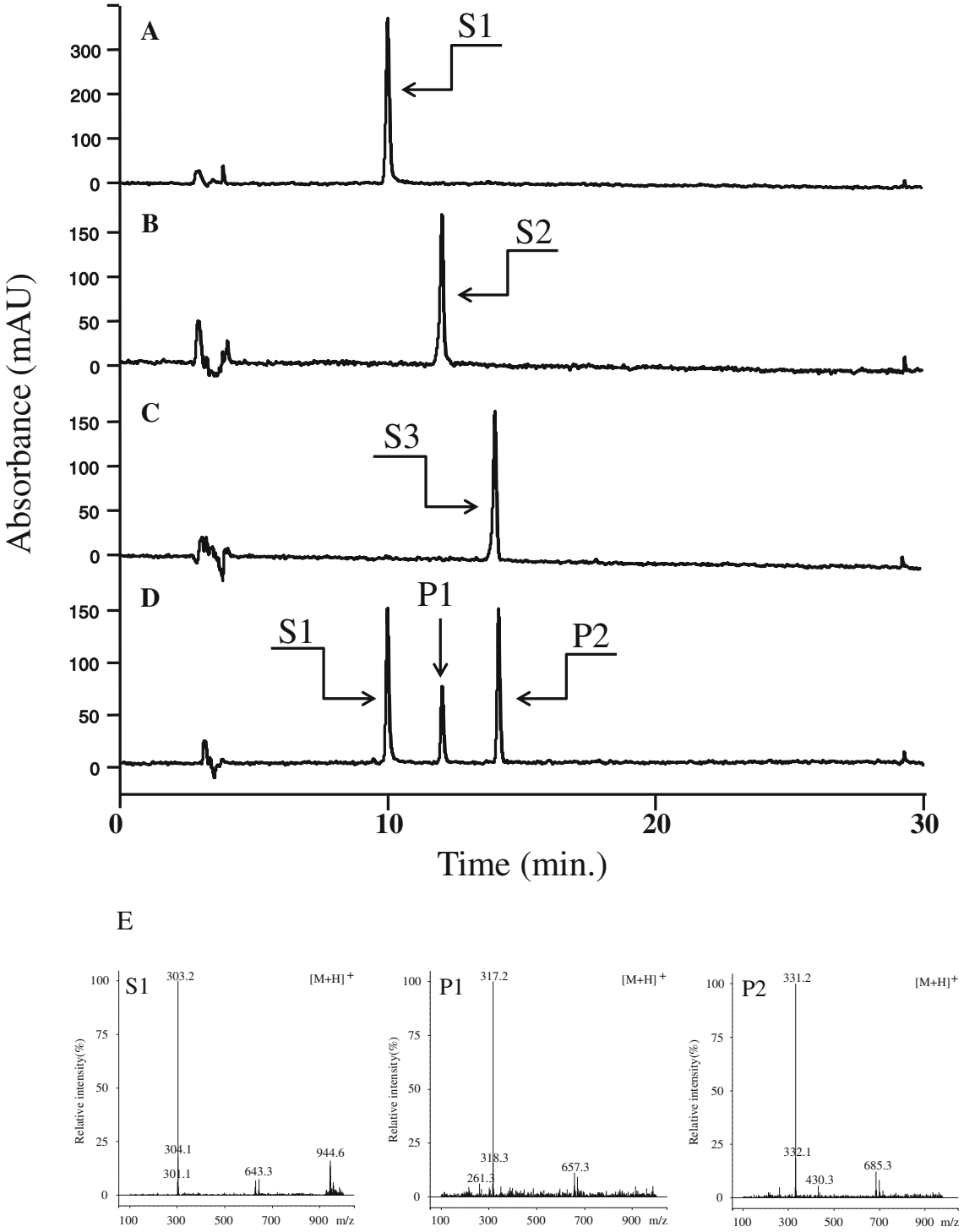

Fig. 2 Analysis of the tricetin reaction product by high-performance liquid chromatography and mass spectra. Authentic tricetin (**a**), authentic selgin (**b**), authentic tricin (**c**), tricetin reaction products (**d**), and MS spectra of reaction product (**e**). The molecular mass of P1 increased 14 Da compared with S1, and the molecular mass of P2 increased 28 Da compared with S1. The mass spectrometer was operated in the positive mode (ESI+)

Table 1 Kinetic parameters of OsOMT26 and OsOMT26 Asp210Glu

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
OsOMT26	Caffeoyl-CoA	48.58 (±2.71)	4.03 (±0.12)	8.33 (±0.04)×10^{-2}
OsOMT26	Caffeic acid	28.53 (±2.51)	1.03 (±0.09)	3.61 (±0.14)×10^{-2}
OsOMT26Asp210Glu	Caffeic acid	86.85 (±2.71)	3.04 (±0.12)	0.35 (±0.04)×10^{-2}
OsOMT26	Luteolin	34.28 (±3.96)	0.46 (±0.05)	1.35 (±0.02)×10^{-2}
OsOMT26	Tricetin	34.06 (±4.30)	0.41 (±0.04)	1.22 (±0.05)×10^{-2}
OsOMT26Asp210Glu	Tricetin	23.31 (±2.51)	0.62 (±0.04)	2.45 (±0.05)×10^{-2}

the production of a product. These three flavonoids contain a 3′-hydroxyl group. The reaction product of luteolin and quercetin contained the same retention time as 3′-*O*-methylluteolin and isorhamnetin (3′-*O*-methylquercetin). Two products were produced when tricetin was used as a substrate. The molecular mass of one of the products was 14 Da heavier than that of tricetin, and the molecular mass of the other was 28 Da heavier than that of tricetin (Fig. 2e). This indicated that the first product was monomethylated and the second one was dimethylated. The monomethylated product had the same retention time as selgin (3′-*O*-methyltricetin), and the dimenthylated product had the same retention time as tricin (3′,5′-*O*-dimethyltricetin; Fig. 2). When the reaction time using tricetin as a substrate was increased, only the dimethylated product was observed. This was similar to ROMT15 and ROMT17 (Lee et al. 2008). OsOMT26 required a metal ion such as Mg^{2+} to be active. Omission of the metal ion resulted in a complete loss of activity.

Kinetic parameters, K_m and k_{cat}, were calculated, and caffeoyl-CoA was found to be the best substrate for OsOMT26, followed by caffeic acid, luteolin, and tricetin (Table 1).

Structural Basis of Reactivity

We then examined the structure of OsOMT26 to understand the reason why caffeic acid and caffeoyl-CoA were better substrates than luteolin or tricetin (Table 1). The three-dimensional structure of OsOMT26 was built using molecular modeling. The overall structure of the OsOMT26 was similar to the template CCoAOMT. Caffeic acid was docked into OsOMT26 because the coenzyme A part of caffeoyl-CoA was so big that it did not fit into the active site of OsOMT26. But the methylation part of caffeoyl-CoA (caffeic acid part) fits properly into the substrate-binding pocket. The docking of caffeic acid or tricetin into the substrate-binding site revealed which amino acids were important to the catalysis. Asp159, Asp190, and Asn191 were coordinated with metal ion. The metal ion was located between SAM and a methyl group acceptor, as has been observed in other CCoAOMT-type OMTs. The metal ion serves as a base to remove a proton from a methyl group acceptor (Cho et al. 2008; Ferrer et al. 2005; Kopycki et al. 2008). As expected, the phenyl group of caffeic acid, which eventually accepts the methyl group from SAM, was positioned toward the SAM group, while the acylic acid part was positioned at a site opposite of SAM. In the case of tricetin, the B ring was located toward the SAM, but the A ring and the C ring were located on the opposite site. Flavonoid OMTs employ two methods to properly position the methyl group acceptor in the substrate-binding pockets: the first is the formation of a hydrogen bond between the amino acid in a substrate-binding pocket and the methyl group acceptor. The other is the formation of a hydrophobic

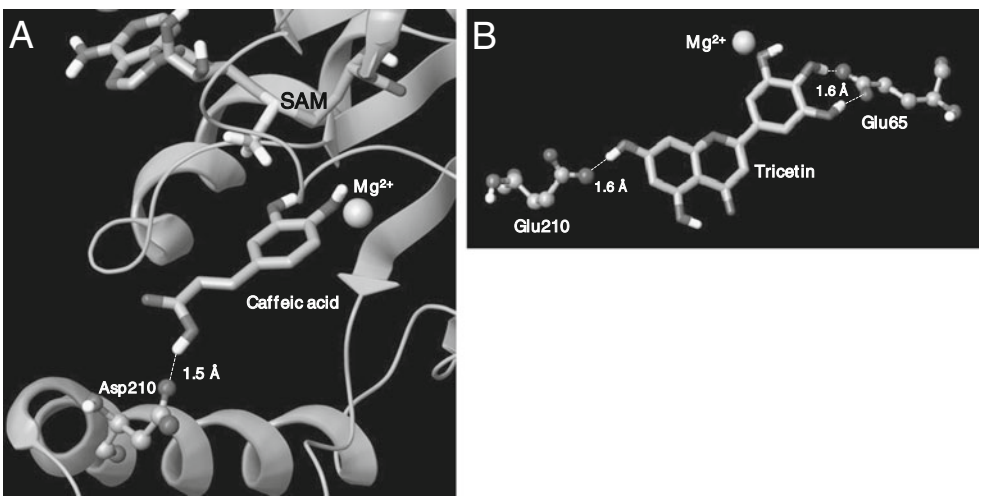

Fig. 3 Molecular docking of caffeic acid (**a**) or tricetin (**b**) onto OsOMT26. Amino acid residues forming a hydrogen bond(s) with the substrate are shown

pocket in which the phenol ring of the methyl group acceptor fits (Kim et al. 2010). In the substrate-binding pocket of CCoAOMT, several amino acid residues interact with the CoA part of the substrate to form hydrogen bonds (Ferrer et al. 2005). PFOMT has a hydrophobic pocket in which the other flavonoid rings fit (Kopycki et al. 2008). Thus, instead of forming a hydrogen bond, hydrophobic interactions are important for the proper positioning of the substrate in the substrate-binding pocket of PFOMT. OsOMT26 uses the first method to place the methyl group acceptor in the right position for the reaction. The distance between Asp210 and the carboxylic acid of caffeic acid was 1.5 Å, which was close enough for the formation of a hydrogen bond (Fig. 3). The 3′ and 4′ hydroxyl groups of tricetin were within an appropriate distance to form hydrogen bonds with Glu65 (Fig. 3). However, the formation of a hydrogen bond between Asp210 and the hydroxyl group of the A ring in tricetin was not favorable due to the following reasons. First, an intramolecular hydrogen bond between the carbonyl oxygen of carbon 4 and the 5-hydroxyl group of tricetin was formed. In order to form a hydrogen bond between Asp210 and the 5-hydroxyl group of tricetin, the intramolecular hydrogen bond needs to be broken. Second, the distance between the Asp210 of OsOMT26 and the 7-hydroxyl group of tricetin was 2.3 Å, which was too far for the formation of a hydrogen bond. Thus, the binding of tricetin to the binding pocket of OsOMT26 was less stable than that of caffeic acid, which explains why caffeic acid was a better substrate than tricetin.

In order to examine the role of Asp210 in the reaction, site-directed mutagenesis was carried out. In the case of caffeic acid, Asp210 was ideally placed for the formation of a hydrogen bond. However, tricetin was not close enough to form an ideal hydrogen bond. In other words, if the distance between the hydrogen acceptor and hydrogen donor was too close, a hydrogen bond cannot be formed. Thus, Asp210 was mutated into Glu. The calculated distance between Glu210 and the carboxylic acid of caffeic acid was 2.8 Å, which was too far for a hydrogen bond to be formed. As predicted, the activity of Asp210Glu mutant toward caffeic acid decreased by 90%. However, the calculated distance between the 7-hydroxyl group of tricetin and Glu210 was 1.6 Å, which was close enough for a hydrogen bond to be formed. The activity of Asp210Glu toward tricetin increased by approximately twofold compared with the wild type, as measured by k_{cat}/K_m. This indicated that the hydrogen bond between the 7-hydroxyl group and Glu was critical for the reactivity of OsOMT26.

OsOMT26 had a higher preference for caffeoyl-CoA than flavonoids, indicating that it is CCoAOMT-type OMT. The architecture of the methyl group acceptor binding site provides an explanation as to why cafferoyl-CoA or cafffeic acid was a better substrate for OsOMT26. The modification of the methyl group binding site improved the reactivity of OsOMT26 toward tricetin. Random mutagenesis such as error-prone polymerase chain reaction was used to change the regioselectivity of OMT (Joe et al. 2010). However, this study showed that structural-based alternation of OMT can improve its reactivity, which is important for the synthesis of specific *O*-methylated phenylpropanoid (Kim et al. 2010).

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