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Comparing the acceptor promiscuity of a *Rosa hybrida* glucosyltransferase RhGT1 and an engineered microbial glucosyltransferase OleD^{PSA} toward a small flavonoid library

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ABSTRACT

Glycosylation is a widespread modification of plant secondary metabolites, and catalyzed by a superfamily of enzymes called UDP-glycosyltransferases (UGTs). UGTs are often involved in late biosynthetic steps and show broad substrate specificity or regioselectivity. In this study, the acceptor promiscuity of a *Rosa hybrid* UGT RhGT1 and an evolved microbial UGT OleD^{PSA} toward a small flavonoid library was probed and compared. Interestingly, RhGT1 showed comparable acceptor promiscuity in comparison with OleD^{PSA}, though the acceptor binding pocket of the latter is much more open and large. This clearly indicates that stabilization of the acceptor position by suitable hydrophobic interactions is important for the specificity or regioselectivity determination as well as overall fit of the acceptor into a 'big enough' binding pocket. This also poses a challenge for structure-based UGT engineering to alter the glucosylation pattern of flavonoids.

1. Introduction

Flavonoids, possessing potentially useful pharmacological and nutraceutical activities, represent a highly diverse class of secondary metabolites with over 8000 different members. In nature, most flavonoids occur in conjugated form with sugar residues. Glycosylation of flavonoids in plant cells usually takes place in a regiospecific manner after the completion of aglycone biosynthesis[1] and is catalyzed by a superfamily of enzymes called UDP-glycosyltransferases (UGTs), which catalyze the transfer of UDP-activated sugar moieties to specific acceptor molecules.[2] Glycosylation is a key mechanism in determining chemical diversity of plant natural products, and also alters the hydrophilicity of the parent compounds, their stability, their subcellular localization, and often their bioactivity.[3,4] UGTs are quite divergent and usually show different acceptor specificity and regioselectivity.

Recent crystallization of four flavonoid UGTs, including UGT71G1,[5] VvGT1,[6] UGT85H2,[7] and UGT78G1,[8] shed light on the structural basis of the substrate specificity and regioselectivity.[9] Their acceptor pockets, mainly consisting of N-terminal residues, are formed by several helices and loops, and all shaped like deep, narrow canyons, whereas the conformations of individual pockets are highly varied (Supplementary Fig. S1). Both UGT71G1 and UGT85H2 possess more open acceptor binding pockets compared with UGT78G1 and VvGT1, and can glucosylate five and two hydroxyls of quercetin, respectively. However, UGT78G1 and VvGT1 only generated one glucosylated product with quercetin as substrate. For UGT71G1, size alteration of the acceptor pocket resulting from introduction of smaller amino acid side chains (F148V or Y202A), dramatically changed the quercetin glucosylation pattern from predominant 3′-*O*-gluside to 3-*O*-gluside. In the meantime, the Y202A mutant gained a novel activity to glucosylate the 5-hydroxyl of genistein.[10] Thus, UGTs possessing more open and large acceptor binding pocket may also have higher promiscuity owing to its spacious acceptor binding pocket offering more plasticity with respect to acceptor positioning. As one investigator summarized, 'acceptor specificity is determined by the overall shape and size of the acceptor pocket'[11] for flavonoid UGTs.

It is worth noting that some microbial UGTs, such as an oleandomycin glucosyltransferase (OleD) involving in the glucosylation and inactivation of macrolide antibiotics in *Streptomyces antibioticus*, can also glucosylate flavonoids.[12] Up to present, six microbial UGTs (OleD, OleI, MGT, BcGT-1, BcGT-3, and XcGT-2), all harboring

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a MGT domain, were reported to be able to glucosylate a variety of flavonoids.[13–16] Compared to the wild type OleD, an evolved triple mutant P67T/S132F/A242V (referred to as OleD^{PSA} here) glucosylated 76 diverse drug-like acceptors more proficiently.[17,18] In contrast with the canyon-like pockets, which seem to be fitted in tightly by diverse planar flavonoids, of flavonoid UGTs from plant, the acceptor binding pockets of OleD and OleD^{PSA} are more like 'huge' cavities (Fig. 1a and b).[19] The more open and large acceptor pocket may endow the host with broader acceptor specificity or regioselectivity. To investigate the validity of the assumption, we compared the substrate promiscuity of OleD^{PSA} and a *Rosa hybrida* UGT RhGT1, which is an anthocyanidin 5,3-*O*-glucosyltransferase,[20] toward 41 representative flavonoids (Supplementary Fig. S2).

2. Results and discussion

2.1. pH profiles of RhGT1 and OleD^{PSA}

RhGT1 is active under mild acid conditions and reach the optimal level of activity at pH 7.0 (Fig. 2a). For OleD^{PSA}, an optimal glucosylation activity against both substrates galangin and 7-hydroxyflavone was observed at pH 8.0 (Fig. 2b), which is consistent with the optimal pH value of wild type OleD with oleandomycin as substrate.[21]

2.2. Homology modeling of RhGT1

Among the four flavonoid UGTs with known structures, UGT71G1 shares highest sequence identity (34%) with RhGT1, and its structure thus was employed as template for RhGT1 modeling. Structural comparison indicated that two molecules are highly similar to each other with a small root mean square deviation (RMSD) of 1.1 Å for 437 Cα atoms. The most diverging regions are loops connecting Nβ2–Nα2 in N-terminal domain and Cβ2–Cα2 in C-terminal domain (Fig. 1c). These two loops in RhGT1 are longer, and the loop Cβ2–Cα2 is positioned closer to the acceptor binding site. The RhGT1 model owns a narrow, deep canyon-like acceptor binding pocket (Fig. 1d), which locates to a similar 3D position compared with that of acceptor binding sites in the four plant UGT structures. For these four flavonoid UGTs, a highly conserved histidine was observed in their active sites, and proposed to act as a general base and catalytic residue for enzyme activity to abstract a proton from the acceptor substrate.[9] Sequence alignment showed that a corresponding His16 is present in RhGT1 (Supplementary Fig. S3). When the histidine was replaced by an alanine, RhGT1 completely lost activity. For OleD^{PSA}, site-directed mutations of H22A resulted in a non-active protein, suggesting His22, which acts as a general base for deprotonation of oleandomycin,[19] functions as same when glucosylating flavonoid acceptors. To eliminate possible visual error in assessing and

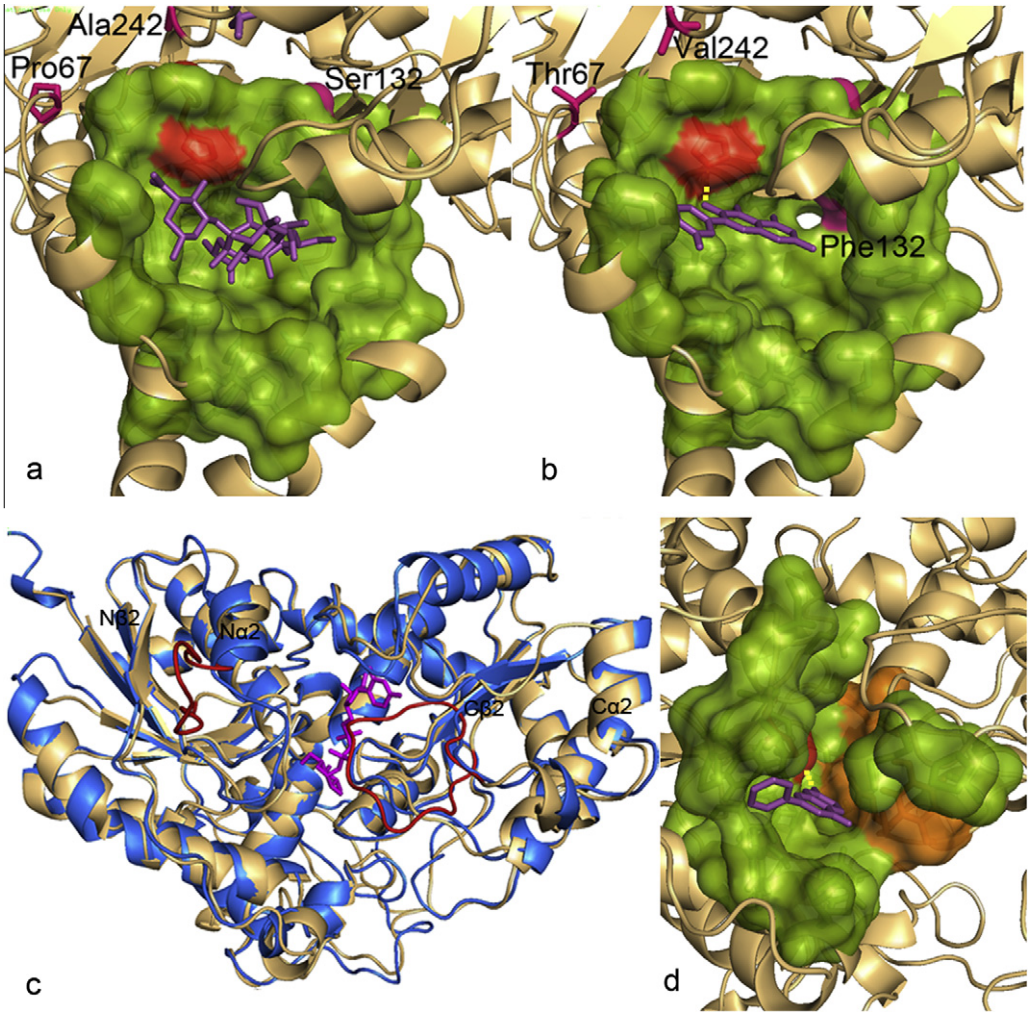

Figure 1. Surface representation of acceptor binding pockets of OleD (a), OleD^{PSA} (b) and RhGT1 (d), and superimposition of RhGT1 model and UGT71G1 structure (c). Erythromycin, quercetin and 7-hydroxyflavone are illustrated as magenta sticks. Potential hydrogen bonding interactions are indicated by dotted lines. Mutation residues in OleD^{PSA} are labeled. The residues within PSPG-box [4] and the putative catalytic residues (His) are shown in orange and red, respectively. The two hypervariable loops in RhGT1 are highlighted in red.

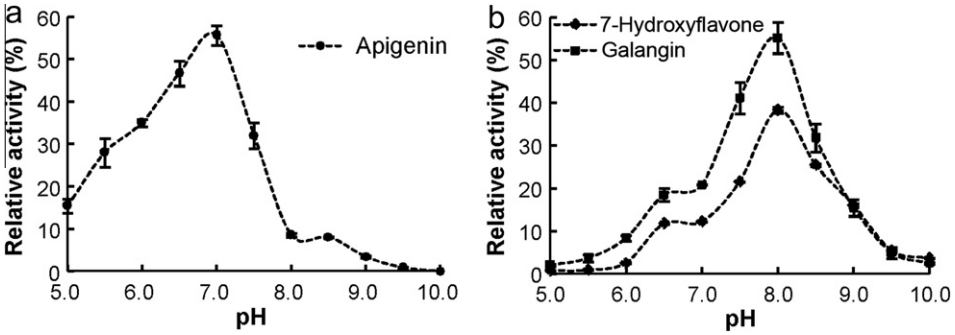

Figure 2. pH profiles of RhGT1 (a) and OleD^{PSA} (b).

comparing the acceptor binding pockets of RhGT1 and OleD^{PSA} with respect to their shape and size, quercetin and 7-hydroxyflavone with similar size were used as references and docked into the binding pockets of OleD^{PSA} and RhGT1, respectively (Fig. 1b and d).

2.3. Comparison of substrate specificity and regioselectivity

To our surprise, RhGT1 showed comparable acceptor plasticity in comparison with OleD^{PSA}. Both RhGT1 and OleD^{PSA} glucosylated a spectrum of diverse flavonoids, including flavones, flavonols, flavanones, isoflavones, and chalcones out of **41** flavonoid compounds (Fig. 3). Apart from **21** common acceptors, **21** and **32** were exclusively glucosylated by RhGT1, while **22** and **24** were accepted only by OleD^{PSA}. Based on the hydroxyl groups available on the flavan backbone structure, the glucosylation could occur at positions C-3, C-5, C-6, C-7, C-3', and C-4′ for both RhGT1 and OleD^{PSA}. In addition, OleD^{PSA} produced additional C-8 or C-2′ glucosides when **7** or **22** was used as substrate. RhGT1 seems more active at position C-6 over C-3 and C-7 in that the conversion rate dropped obviously when **1** or **3** substituted **2** as acceptor substrate. In contrast, OleD^{PSA} is more efficient to glucosylate **1** and **3** but **2**.

Double bond between the C-2 and C-3 atoms in the C ring seems to play an essential role in the catalysis and the regioselectivity for both UGTs. Flavonoids **4**, **6**, **21**, **26**, and **27** differ from **3**, **5**, **2**, **12**, and **13** by the absence of a double bond between two carbon atoms (C2–C3), respectively. Flavanone **4** was preferred for both RhGT1 and OleD^{PSA} to flavone **3**. In contrast, none of **26** and **27** was accepted by either RhGT1 or OleD^{PSA}, while **12** and **13** were converted into their corresponding monoglucosides by both enzymes. Compound **6** was glucosylated more efficiently by OleD^{PSA} than **5**, though both of them were converted into their glucosides by RhGT1 without considerable difference in yield rate. Compound **2** is an excellent substrate for RhGT1 (100% conversion rate) and also accepted by OleD^{PSA}, whereas its flavanone form 21 was glucosylated with a lower yield rate 69% by RhGT1 and is not an acceptor of OleD^{PSA}.

Regioselectivity of RhGT1 and OleD^{PSA} appeared to be affected by the overall structure of flavonoids not just the site of an accepting hydroxyl group regardless of the difference of their acceptor

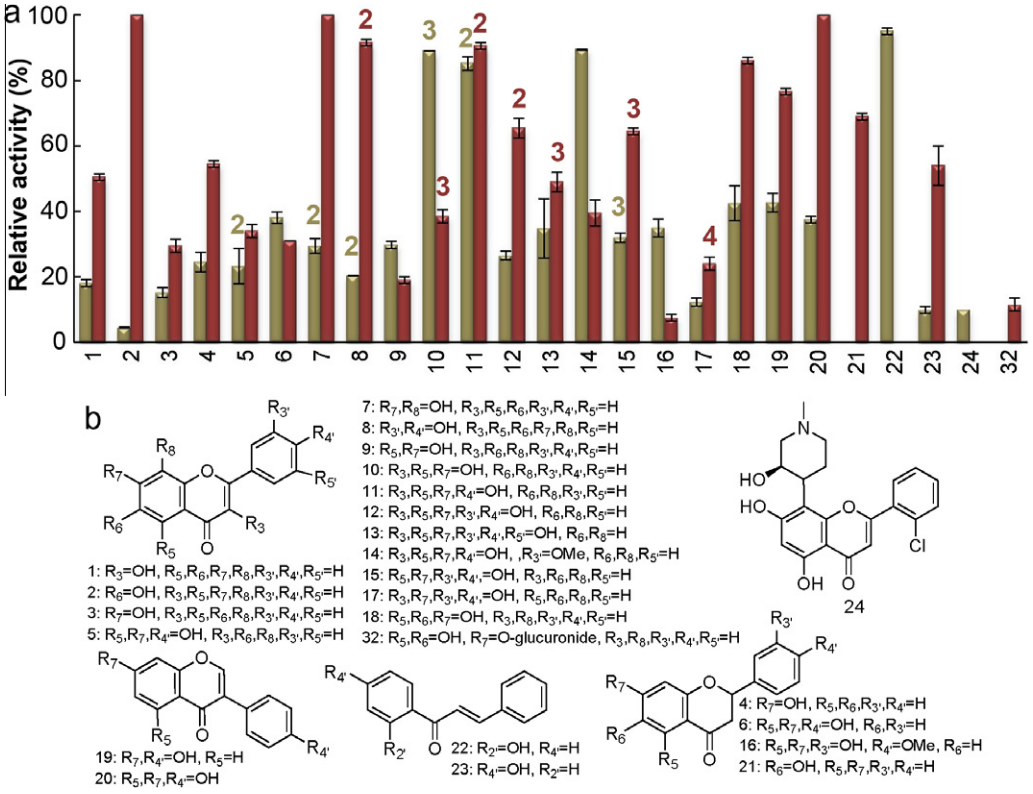

Figure 3. The acceptor specificity (a) of RhGT1 (dark red) and OleD^{PSA} (light brown) toward 25 flavonoids (b). Numbers above bars indicate how many different products are formed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

binding pockets in shape or size. For example, **8** and **10** offer two and three free hydroxyl groups, respectively, and were converted into two and three different monoglucosides by both RhGT1 and OleD^{PSA}. Compound **12** happens to carry all the five hydroxyl groups for potential O-glucosylation sites, but OleD^{PSA} is only capable of glucose transfer to the hydroxyl group of C-3 on the C ring (Fig. 1b). The glucosylation position was determined by an NMR analysis (see the Supplementary data). Likewise, when **12** was used as substrate of RhGT1, only two glucoside products formed. Moreover, **25** was totally not accepted by either RhGT1 or OleD^{PSA}, though it offers five free hydroxyl groups and differs from its isomer **12** only with a hydroxyl group located at the C-2′ in B ring. The deduction was evidenced as well by the diverse glucosylation patterns generated by a set of flavones **9**–**13** (Fig. 3). Compounds **10**–**13** carry one additional hydroxyl group sequentially at positions C-3, C-4′, C-3′, and C-5′ on the basis of **9**–**12** (Fig. 3b).

RhGT1 and OleD^{PSA} also showed different plasticity toward flavonoids decorated with methoxy moiety. For example, the methyl decoration of **14** and **16** at C-3′ and C-4′, respectively, made them bad acceptors for RhGT1, but OleD^{PSA} can tolerate this modification very well. The fact suggested that the methoxyflavanones **14** and **16**, even with a bigger structure size compared to their isomers **12** and **15**, could still fit into the large cavity-like acceptor pocket of OleD^{PSA} in a favorable manner. In brief, RhGT1 and OleD^{PSA} showed broad acceptor specificity toward flavonoid aglycones (**1**–**31**), since both of them can glucosylate compounds **1**–**20** and **23**. In addition, **21** and **22** are acceptors of RhGT1 and OleD^{PSA}, respectively. Flavone **30** with one free hydroxyl group at C-5 position was not accepted by both UGTs, even though **10** can be catalyzed to a C-5 monoglucoside by both enzymes. Compound **31** (Procyanidin), owning a much larger structural size in comparison with other flavonoid aglycones, was not glucosylated by both UGTs either.

To our surprise, ten flavonoid glycosides including six monoglycosides and four diglycosides used in this study are not acceptors for OleD^{PSA}, and only **32** (baicalin) out of the 10 compounds was tolerated by RhGT1 in spite of a low conversion rate (12%). However, when excessive UDP-Glc (20 equiv) was provided, **10** was almost quantitatively converted into three different monoglucosides by OleD^{PSA} in 1 h, then one of the monoglucosides was further converted into diglucoside (Fig. 4). More intriguingly, **24** (flavopiridol), a semisynthetic flavonoid that potently inhibits various Cyclin-Dependent Kinases (CDKs) and displays unique anticancer properties,[22,23] was exclusively glucosylated by OleD^{PSA} but RhGT1. Compounds **42**, **43**, and **44**, which are non-flavonoid glucosides, were not accepted by both enzymes. Both size and hydrophobicity can be significantly altered when these aglycones were decorated with Glc or Glucuronic acid moiety. Therefore, how the decoration resulted in poor tolerance observed for both UGTs toward flavonoid glycosides (**32**–**41**) could be very profound.

Some UGTs were reported to catalyze both deglycosylation and reversible reaction; the latter allows sugars and aglycones to be exchanged.[24] However, neither RhGT1 nor OleD^{PSA} exhibited deglycosylation or reversible reaction in this study.

Though the broad substrate promiscuity exhibited by most UGTs in vitro would pose a question how the specificity of these enzymes is controlled in vivo, broad specificity is extremely useful in altering the glycosylation pattern of natural product which is considered as a source of drug leads. Recently, several UGTs including AtUGT78D3,[25] UGT73B2,[26] ArGt-4,[27] and the wild type OleD[12] et al., have been employed for glucosylation of natural or modified flavonoids in *Escherichia coli*. With respect to the low acceptor specificity showed by RhGT1 and OleD^{PSA}, either of them represents an interesting enzyme candidate for engineering flavonoid diversity.

In summary, flavonoids **1**–**20** and **23** are common acceptors for both RhGT1 and OleD^{PSA}, while compounds **21** and **32**, and compounds **22** and **24** were exclusively glucosylated by RhGT1 and OleD^{PSA}, respectively. Both UGTs showed broad acceptor specificity, though the acceptor binding pocket of OleD^{PSA} is much more open and large. This clearly indicates that stabilization of the acceptor position by suitable hydrophobic interactions is important for the specificity determination as well as overall fit of the acceptor into a 'big enough' binding pocket. UGTs may require multiple amino acids to provide the suitable hydrophobic interactions. At this point, structure-based UGT engineering for manipulation of glucosylation is still a challenging job.

3. Experimental

3.1. Expression and purification of the recombinant RhGT1 and OleD^{PSA}

Both *rhGT1* and *oleD^{PSA}* were cloned from synthetic genes whose codons were optimized for *E. coli* expression system. A truncated OleD^{PSA} having a C-terminal 23-amino-acid deletion was expressed as an N-His6-tagged protein and purified to homogeneity as described previously.[18] The recombinant RhGT1 was expressed as a C-His-tagged protein in *E. coli* BL21 (DE3) and purified by Ni^{2+}-affinity chromatography. Mutagenesis was performed using the fast site-directed mutagenesis kit (TransGen Biotech, Beijing, China). Mutations were confirmed by sequencing.

3.2. pH Profiles by HPLC

Assays were performed in a total volume of 100 μL in a buffer (100 mM) with pH varying from 5.0 to 10.0 containing 14 mM β-mercaptoethanol, 20 mM $MgCl_2$, 0.2 mM of various flavonoid substrates, 1 mM UDP-Glc (5 equiv), and a suitable amount of

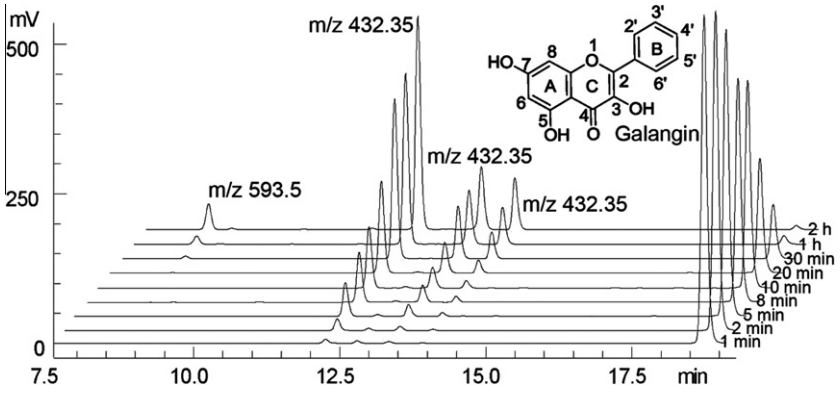

Figure 4. The time course of OleD^{PSA}-catalyzed glucosylation reaction with 10 (galangin) as substrate. Diglucoside is generated from 30 min. Products were identified by LC–MS.

enzymes (84 μg RhGT1 or 30 μg OleD^{PSA}). The buffers used were MES, pH 5.0–6.5; MOPS, pH 7.0; Tris–HCl, pH 7.5–9.0; CAPSO, pH9.5; and CAPS, pH 10.0. After 2 h of incubation at 37 °C, reactions were quenched by the addition of trichloroacetic acid, and products were analyzed by a Shimadzu LC-2010A system equipped with a SPD-20A UV detector and a Synergi 4u Polar-RP$_{18}$ column. Flavonoid glucosides were separated using a linear gradient from 15% to 90% acetonitrile with a flow rate of 1 ml min^{-1} for 30 min. All assays were carried out in duplicate.

3.3. Molecular modeling

UGT71G1 shares 34% sequence identity and 54% sequence similarity with RhGT1, and its structure was used as template for homology modeling of RhGT1. MODELLER was employed to construct the 3D model of RhGT1 via (PS)[2] (pronounced PS square) homology modeling server (http://ps2.life.nctu.edu.tw/). The model was optimized by several steps of energy minimization of side chains and loop regions. Stereochemical and overall quality of the final model was assessed by using PROCHECK and ProSA. No residues were located in disallowed regions, and only seven residues in the model were in generously allowed regions. The PROCHECK overall g factor evaluating all torsion angles and bond lengths was −0.11, indicating a high-quality model. The ProSA Z score of the RhGT1 model was calculated to be −10.07. Z scores for experimentally determined X-ray structures of proteins with similar size (around 450 amino acids) lie in the range of −6 to −13. The Z score of the model was thus within the range of scores typical for native proteins with similar size. The structure model of OleD^{PSA} was generated by CPHmodels[28] with OleD structure as template. Docking quercetin (or 7-hydroxyflavone) into the putative binding pocket of OleD^{PSA} (or RhGT1) was performed by using autodock4.2.[29]

3.4. Substrate specificity studies

Flavonoid substrates were purchased from Alfa aesar (Tianjin, China), Meryer (Shanghai, China) and Aladdin (Shanghai, China), and dissolved in DMSO. Enzyme assays were performed in 100 mM MES or Tris–HCl (pH 7.0 for RhGT1 and pH 8.0 for OleD^{PSA}) as above described. Products quantitatively converted or multiple products generated in one reaction were identified by LC–MS (Table S1).

3.5. Mass spectrometry (LC–MS)

LC–MS analysis of the flavonoid glucosides was carried out on a LC–MS 2020 (Shimadzu) system. Identification was based on a combination of HPLC retention time, UV, and mass spectrometry spectral data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2012.12.012.

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