Structural Insights into the Catalytic Mechanism of a Plant Diterpene Glycosyltransferase SrUGT76G1

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ABSTRACT

Diterpene glycosyltransferase UGT76G1 from Stevia rebaudiana (SrUGT76G1) is key to the generation of economically important steviol glycosides (SGs), a group of natural sweeteners with high-intensity sweetness. SrUGT76G1 accommodates a wide range of steviol-derived substrates and many other small molecules. We report here the crystal structures of SrUGT76G1 in complex with multiple ligands to answer how this enzyme recognizes diterpenoid aglycones and catalyzes the 1,3-sugar chain branching. A spacious pocket for sugar-acceptor binding was observed from the determined SrUGT76G1 structures, which can explain its broad substrate spectrum. Residues Gly87 and Leu204 lining the pocket play key roles in switching between diterpenoid and flavonoid glucosylation. An engineered mutant of SrUGT76G1, T284S, could catalyze a selectively increased production of next-generation sweetener rebaudioside M, with diminished side product of rebaudioside I. Taken together, these results provide significant insights into molecular basis of the substrate specificity of scarcely documented diterpenoid glycosyltransferases and would facilitate the structure-guided glycoengineering to produce diversified diterpenoids with new activities.

Key words: glycosyltransferase, diterpenoid, crystal structure, substrate specificity, enzyme engineering

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INTRODUCTION

The kaurane-type diterpenoids, a large and important group of terpenes, have been found to be widespread in plants, including Labiatae, Compositae, and Euphorbiaceae. Many kauranoid derivatives of natural origin have been reported as active principles for anti-HIV (Chang et al., 1998), anti-inflammatory (Kuo et al., 2013), antibacterial (Cai et al., 2017), and antitumor drugs (Hu et al., 2018). However, only a few plant species have been reported as sources of diterpene glycosides with kaurane skeleton (Ceunen and Geuns, 2013). Expansion of the diterpenoid glycosides pool and customized modification are essential for candidate drug discovery and molecular function improvements. However, this is hampered by the limited knowledge of the molecular basis of diterpenoid glycosylation.

Steviol glycosides (SGs) are well-known diterpenoid glycosides bearing multiple glyosyl substitutes attached to C13 and C19 of ent-kaurene aglycone. The major SGs, such as stevioside and rebaudioside A (RebA), are 300 times sweeter than sucrose (Hellfrisch et al., 2012; Prakash et al., 2014; Olsson et al., 2016) and have been commercialized as natural non-caloric sweetener for decades. Recent development of next-generation sweetener covers numerous natural glycosides including rebaudioside D (RebD) and rebaudioside M (RebM), which are rare naturally occurring SGs with less than 0.4%–0.5% (w/w) in plants (Olsson et al., 2016). The major SGs biosynthetic pathway in Stevia rebaudiana has been shown to involve four uridine diphosphate (UDP)-dependent glycosyltransferases (UGTs) consecutively conjugating up to six sugar moieties to the steviol skeleton (Ceunen and Geuns, 2013) (Supplemental Figure 1).

SrUGT76G1 has attracted much attention for its important role of adding C30-glucosyl on stevioside to give RebA, as well as generating the next-generation sweetener RebM from RebD (Supplemental Figure 1, red bold arrows). In addition,
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SrUGT76G1 was reported as a promiscuous UGT catalyzing 1,3-glucosylation on other SGs, such as steviolmonoside (Sm), steviolbioside (Sb), rubusoside (Rub), RebA, rebaudioside E (RebE), and rebaudioside G (RebG) (Olsson et al., 2016). Moreover, 21 small molecules from different natural product classes are also accepted by SrUGT76G1 (Dewitte et al., 2016). Thus, SrUGT76G1 is not only capable of recognizing different diterpene skeletons to produce branch sugar chains but also holds great potential to diversify various classes of natural glycosides.

Some plant-derived UGT structures have been dissolved, with most attention focused on the interactions between the enzyme and the sugar donor. However, few studies have investigated how the sugar acceptors can be specifically distinguished by their own glycosyltransferases. For example, the structure complex of multifunctional triterpene/flavonoid glycosyltransferase MtUGT71G1 with UDP and UDP-glucose provided the first insight into the glycosylation mechanism that involves His22 as the catalytic base and Asp121 as key residue (Shao et al., 2005). In the cases of VvGT1 from Vitis vinifera (Offen et al., 2006) and UGT78K6 from Clitoria ternatea (Hiromoto et al., 2015), the flavonoid-recognizing pockets in the N-terminus of these UGTs were described. Recently, the structure of Os79 from Oryza sativa has been reported in complex with UDP-2-fluoro-2-deoxy-D-glucose and trichothecene, but few features of acceptor binding were characterized due to the weak electron density of acceptor substrate analogs in Os79 (Wetterhorn et al., 2016). How glycosyltransferases tolerate substrates with varying sizes and the molecular basis for UGTs to specifically recognize different aglycones remain to be elucidated.

Here, we determined four structures of SrUGT76G1 in complex with different ligands, including both sugar acceptors and donors. Through analysis of these structures, we can snapshot four different statuses of SrUGT76G1, namely donor and acceptor binding, glycosylation, glycosylated product formation, and product release. Based on the knowledge obtained from structures, we engineered SrUGT76G1 to investigate influential residues in determining substrate and product preference. Altered substrate and product spectra of SrUGT76G1 were achieved by narrowing or expanding the acceptor pocket, which diversified the diterpene structures.

RESULTS

Overall Structure of the Diterpenoid Glycosyltransferase SrUGT76G1

To verify the function of SrUGT76G1, we systematically tested eight available ent-kaurene type substrates through in vitro reactions (Supplemental Figure 2). SrUGT76G1 was found to act on ent-kaurenoïds bearing at least one glucosyl on C13-hydroxyl or C4-carboxyl but did not accept the non-sugar-bearing aglycone steviol and kaurenoic acid (Figure 1). The affinity and catalytic efficiency of SrUGT76G1 toward seven different substrates were quantified (Supplemental Table 1). The kinetic parameters (Sm versus Sb, RebA versus RebD) indicate that SrUGT76G1 adds a third glucosyl at the C3’ position of sophorosyl (1,2-diglucosyl) more easily in comparison with monoglucosyl moieties without C2’-glucosyl. The $k_{cat}/K_M$ of steviolbioside is nearly 7 times that of steviolmonoside, while that of RebD is more than 10 times that of RebA (Supplemental Table 1).

To understand the molecular basis of why SrUGT76G1 recognizes a vast set of diterpenoid substrates and shows different catalytic efficiency, we crystallized SrUGT76G1 complexed with UDP (UDP-SrUGT76G1), UDP-xylose (UDPX-SrUGT76G1), UDP and steviolmonoside (UDP-Sm-SrUGT76G1), and UDP and steviolbioside (the structure determined turned out to be the product rebioside B-SrUGT76G1 complex; UDP-RebB-SrUGT76G1), and determined their structures to 2.6-Å, 2.5-Å, 2.4-Å, and 2.0-Å resolution, respectively (Figure 2A–2D). Data collection, phasing, and refinement statistics are presented in Supplemental Table 2.

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Structure of Glycosyltransferase SrUGT76G1

Through comparison of the structures, we found that the UDP molecule in the UDP-SrUGT76G1, UDP-Sm-SrUGT76G1, or UDP-RebB-SrUGT76G1 structure superimposed well with the UDP-xylose molecule in the UDPX-SrUGT76G1 structure, except for an extra xylose moiety (Figure 3A–3D), all located in a large catalytic pocket between the NTD and CTD. The catalytic pocket is fit for the sugar-acceptor substrate Sm or RebB in addition to the donor substrate, and the glucosyl moiety of sugar donor can transfer its glucosyl moiety to Sm or RebB, forming a β-1,3-O-glicosidic bond. By comparing the structures of UDP-RebB-SrUGT76G1 and UDP-Sm-SrUGT76G1, the result suggested that the reaction could proceed only when the sugar acceptor has the first glucosyl moiety and the pocket is able to accommodate the sugar acceptor that has the first glucosyl moiety (C13-O-glucosyl) or the first and the second glucosyl moiety (C13-O,1,2-diglucosyl) (Figure 3D). To comprehend the conformational change induced by sugar donor and acceptor binding, we compared and analyzed the structures of UDP-SrUGT76G1 and UDP-RebB-SrUGT76G1. The conformation of the CTD is most likely the same while that of the NTD undergoes a large shift before and after binding substrates. Meanwhile, two disordered regions were found distinctly in the UDP-SrUGT76G1 complex structure: the first region includes residues 165–175 and spans the LR region connecting the NTD and CTD, whereas the second region contains residues 70–73 in the NTD (Figure 3E). As mentioned above, the binding of sugar acceptor has a minor effect on the

Substrate Binding of SrUGT76G1: Conformational Changes and Pocket Volume

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conformation of CTD, which is responsible for sugar donor binding (Figure 3E). Before the sugar-acceptor binding, SrUGT76G1 adopts an open conformation ready to take the sugar acceptor, as represented by the UDP-SrUGT76G1 structure whose LR region and some places of the NTD show an unstable status. After the sugar-acceptor binding, the hydrophobic pocket for diterpene skeleton binding has an obviously conformational change, especially in the $\alpha_2$ helix and $\alpha_8$ helix, which underwent an approximately 5° rotation during sugar-acceptor binding and release (Figure 3F).

Since all the diterpenoids accepted by SrUGT76G1 bear at least one glucosyl, the acceptor-binding pocket was supposed to be much bigger than those of flat compounds such as flavonoid and salicylic acid. To compare the pocket size of VvGT1 (PDB: 2C1Z) (Offen et al., 2006), Os79 (PDB: 5TMD) (Wetterhorn et al., 2016), UGT72B1 (PDB: 2VCE) (Brazier-Hicks et al., 2007), and UDP-RebB-SrUGT76G1 obtained in this research, we computed the volume of substrate-binding pockets of these four structures using CAVER Analyst 2.0 (Jurcik et al., 2018). The volume of the pockets for flavonoid/sesquiterpene glucosyltransferases (2C1Z: 1953.4 Å$^3$; 5TMD: 2288.2 Å$^3$; 2VCE: 1566.4 Å$^3$) are dramatically smaller than that of SrUGT76G1 (3331.1 Å$^3$) (Figure 4). This spacious binding pocket well explained why SrUGT76G1 could tolerate large-sized natural products such as SGs containing multiple sugar substitutes.

Figure 1. A Network of SG Biosynthesis.
Nine SrUGT76G1-catalyzing reactions are indicated by red arrows. The bold arrows indicate a major in planta route to form rebaudioside M (RebM). The diterpenoid aglycones are indicated in gray. The structure of steviol is shown on the upper right. Blue circles represent C13-O-linked glucosyl (Glc1), yellow circles represent C19-O-linked glucosyl (Glc1'), green circles represent 1,2-O-linked glucosyl (Glc2/Glc2'), and red circles represent 1,3-O-linked glucosyl (Glc3/Glc3'). Biochemical validation of SrUGT76G1-catalyzing reactions is shown in Supplemental Figure 2.
Diterpene Skeleton Recognition and Specific Residue Engineering

In the structure of UDP-RebB-SrUGT76G1, the sugar acceptor diterpenoid binds in a large hydrophobic pocket (Figure 5A). This pocket is mainly formed by residues Leu85, Met88, and Ile90 of \( \alpha \)2 helix and Ile199, Leu200, and Ile203 of \( \alpha \)8 helix (Figure 5B). Mutations of these residues are supposed to fine-tune the pocket, which likely influences the substrate preference. To verify this, we designed SrUGT76G1 mutations and determined their relative activities in vitro (Figure 5C). The glycosylation efficiency of L85V and I199L mutations was generally increased in seven tested substrates, while I203L mutation showed a decreased conversion rate. Clustering of substrate types indicate that Sm and Rub, whose C13-O-glucosyl were glycosylated, were less converted in most mutants than in wild-type SrUGT76G1. Mutation of Leu200 to Val selectively enhanced the glycosylation activity on RebA and S19G to give C19-O,1,3-diglucoside products. Mutation of Ile203 to Val specifically increased the conversion rate of RebD and Sb, adding the third sugar on C13 or C19-O-linked sophorosyl groups (Figure 5C).

We further noticed that some other residues such as Gly87 and Pro91 of \( \alpha \)2 helix, and Leu204 of \( \alpha \)8 helix, might also participate in the recognition of diterpene aglycone (Figure 5B). By detailed comparison with reported UGT structures, we found that the residues in corresponding locations of flavonoid-recognizing glycosyltransferases (i.e., MtUGT71G1, MtUGT85H2, VvGT1) were usually large side-chain residue Phe. Residue Ile199 in SrUGT76G1 also corresponded to some Phe in flavonoid UGTs. We therefore considered that mutation of Gly87, Pro91, Ile199, and Leu204 to Phe might narrow the substrate-binding pocket to fit flavonoid recognition (Figure 5D). Four Phe mutants were generated and tested toward a flavone \( C \)-glycoside “isoorientin” in vitro. Unlike the wild-type SrUGT76G1 which only has feeble activity, G87F, I199F, and L204F mutants showed dramatic enhancement in the glycosylation of isoorientin into two glycosylated products (Figure 5E). Meanwhile, the activity of G87F and L204F mutants toward diterpenoids (for example, steviolbioside) decreased significantly, suggesting that these residues play an important role in switching diterpenoid/flavonoid recognition (Figure 5F).

Key Residues Involved in Sugar Recognition Aid Branch Chain Formation

The UDP-RebB-SrUGT76G1 structure clearly shows the specific recognition of the glucosyl moiety (Glc1–Glc3) (Figure 6A and 6B). Residue Thr284 forms two hydrogen bonds with the oxygen at C4 of Glc1 and the oxygen at C2 of the third glucosyl moiety (Glc3) through a water molecule. Residues Ser147 and His155 stabilize the second glucosyl (Glc2) at C2 position of Glc1, forming hydrogen bonds with the oxygen at C3 and C4 of Glc2. In addition, residue Thr146 forms a hydrogen bond with the oxygen at C6 of Glc3 (Figure 6B). The catalytic base His25 forms two hydrogen bonds with the oxygen at C3 of Glc1 and the oxygen at C2 of Glc2. We proposed that residues Thr284, Ser147, and His155 coordinate to fix the sugar (Glc1 and Glc2)
of the acceptor substrate while Thr146 helps to anchor the added glucosyl (Glc3) after glycosylation.

To verify this assumption, we attempted to mutate these residues and determined their glycosylation specificity through enzymatic assays. According to the previous established model of Glc2 recognition (Lee et al., 2019), replacement of conserved His155 with a large side chain (H155R and H155W) or Ala both weaken the binding of Glc2, which result in decreased activity toward Glc2-containing substrates such as stevioside. Our mutation of His155 to Ala and introduction of a large side chain (H155Y mutation) supported this model, as conversion of Sb (which contains Glc2substantially decreased

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**Figure 3. Snapshot of Differing Ligand-Binding Status and Conformation Change.**

SrUGT76G1 structures accommodating different substrates were superposed. (A) Superposition of UDP-SrUGT76G1 and UDPX-SrUGT76G1 revealed the location of activated sugar; (B) superposed UDPX-SrUGT76G1 and UDP-Sm-SrUGT76G1 represent a state when sugar acceptor is coming close to sugar donor; (C) superposed UDPX-SrUGT76G1 and UDP-RebB-SrUGT76G1 represent a state when glucosylated product is formed; (D) superposition of UDP-RebB-SrUGT76G1 and UDP-Sm-SrUGT76G1 revealed the location of C2-linked glucose; (E) two disordered regions (black spot circles) in UDP-SrUGT76G1 complex structures; (F) binding of sugar acceptor has a major effect on the conformational change of NTD. Magenta, UDP-SrUGT76G1; orange, UDPX-SrUGT76G1; green, UDP-Sm-SrUGT76G1; blue, UDP-RebB-SrUGT76G1.
recycling of UDP-glucose) (Supplemental Figure 5) and transferred forming the acceptor pocket determine the specificity of It has been proposed that amino acids involved in shaping and Residues Restrain the Substrate-Binding Pocket (Figure 6D). Other S147 mutants (S147A and S147N) were also while retaining the ability to accept non-Glc2-containing Sm that completely abolishes the recognition of Glc2-containing Sb (Figure 6C). Glycosylation of non-Glc2-(Figure 6C). Variants of His155 also display an attenuated activity to stabilization role of Thr146 was demonstrated as mutation of Thr146 into Ser or Ala, which slightly impaired the enzyme activity, while the T146N mutation abolished the enzyme activity (Supplemental Figure 4). Th284 is key for 1,3-glucosylation, since a T284A mutation decreased activity in all tested substrates. Interestingly, we found that T284S mutant enhanced the conversion of RebD to RebM while reducing the production of side product rebaudioside I (Rebi) from RebA (Figure 6E). Therefore, we applied this mutant to manufacture RebM using RebA as raw material. To achieve two steps of glycosylation from RebA to RebM, we assembled a dual UGT cassette (P_{T7-EUGT11-SrUGT76G1}) with AtSUS3 (for recycling of UDP-glucose) (Supplemental Figure 5) and transferred it into Escherichia coli BL21(DE3) (strain 651). By this strain, RebA was converted into three products consisting of 70% RebD, 18% RebM, and 12% RebI (Figure 6F). Replacing wild-type SrUGT76G1 with T284S mutant (P_{T7-EUGT11-SrUGT76G1_T284S}, E. coli strain 830, Supplemental Figure 5) dramatically increased the proportion of highly valuable RebM in the fermentation extract with a ratio of RebD/RebM = 1:7. The undesired side product Rebi was completely eliminated (Figure 6F).

Residues Restrain the Substrate-Binding Pocket

It has been proposed that amino acids involved in shaping and forming the acceptor pocket determine the specificity of and Leu379, are located on the two sides of the RebB molecule, where Leu126 forms specific hydrophobic interactions with Glc2 of RebB and Leu379 forms hydrophobic interactions with both Glc2 and the diterpene skeleton of RebB (Figure 7A). Thus, we inferred that Leu126 and Leu379 are probably related to proper orientation of substrates.

To verify this hypothesis, we designed a series of side chains with varying sizes of Leu379 and Leu126 mutations. The enzymatic assay demonstrated that L126V decreased the activity while L126F selectively increased the activity on Sb, stevioside, and RebD (Figure 7B), suggesting that L126F favors 1,3-glycosylation of substrates possessing sophorosyl. Catalytic activity improvement may result from space filling by Phe, as Phe is obviously larger than Val and the importance of space filling caused by single amino acid residues has been reported (Masada et al., 2007). Therefore, Leu126 functions directly in stabilization of the Glc2-containing substrates. We also tested the activity of L379I, L379V, L379W, and L379F mutations, among which L379I showed better activity toward all substrates, especially RebB, while 379F and L379W showed an opposite influence on the tested substrates. In general, the alteration of catalytic activity is complicated and variable for the Leu379 mutations, probably due to the hydrophobic interactions between Leu379 and Glc2 and the diterpene skeleton of RebB (Figure 7B). Our data support that residues forming the acceptor pocket influence the specificity of acceptor substrate.

DISCUSSION

To date, more than 10 structures of plant glycosyltransferases have been reported (Shao et al., 2005; Offen et al., 2006; Brazier-Hicks et al., 2007; Li et al., 2007; Hsu et al., 2015; Wetterhorn et al., 2016; Thompson et al., 2017; Hsu et al., 2018; Zong et al., 2019). However, most of the studies have been focused on the recognition of sugar donors, with few pertaining to the
recognition of sugar acceptors of different classes. As one of the most promiscuous enzymes, SrUGT76G1 provides a representative case for the study of donor–acceptor interaction as well as specific acceptor recognition.

During the preparation of this article, two studies led by Lee and Yang reported the structures of SrUGT76G1 in complex with UDP, RebA, and Rub (Lee et al., 2019; Yang et al., 2019). In UGT76G1-UDP-RebA complex structures (PDB: 6O88 and 6INI), the C19-O-glucosyl ester part of RebA is invisible, which therefore actually represents the structure of RebB. A disordered region in LR is obvious in 6O88 (Supplemental Figure 6A and 6B). In addition, there is some nonspecific binding of glycerol and RebB outside the pocket of UDP-RebA (PDB: 6INI) and UDP-Rub (PDB: 6INH) structures (Supplemental Figure 6B and 6C). Binding of glycerol molecule inside the acceptor pocket of 6INH was also observed (Supplemental Figure 6D). A 30° rotation of steviol aglycone was found between UDP-RebA (PDB: 6INI) and UDP-Rub (PDB: 6INH), while in our structures of UDP-Sm-SrUGT76G1 and UDP-RebB-SrUGT76G1, the diterpenoid aglycone rings were well overlapped. A lack of specific hydrogen bond recognition was considered to explain the rotation in the previous report (Yang et al., 2019), but there is also a possibility that this rotation was caused by the additional glycerol molecule.

In fact, few features of the acceptor specificity determined by the sugar-acceptor pocket are known. He et al. (2006) reported that mutation of Medicago truncatula glycosyltransferase UGT71G1 Phe148 to Val, or Tyr202 to Ala, drastically changed the regioselectivity for quercetin glycosylation from predominantly the 3-O position of the B ring to the 3-O position of the C ring. Additionally, the Y202A mutant gained the ability to glycosylate the 5-hydroxyl of genistein (He et al., 2006). Cartwright et al. (2008) developed an Arabidopsis thaliana UGT74F1 mutant N142Y, which led to formation of the 4-O-glucoside of quercetin. These plant UGT mutation sites were on N5 loop or Nα5, and finally introduced regioselectivity alteration for acceptor substrates. However, bacterial glycosyltransferase crystal structures and mutations indicate that the N3-Nα3 region engaged in determining acceptor specificity. The Streptomyces antibioticus glycosyltransferases, OleI and OleD, glycosylate oleandomycin and diverse macrolides including erythromycin, respectively. The crystal structures of these enzymes show that acceptor difference was caused by structure variation in two regions, one of them being the N3-Nα3 region (Bolam et al., 2007). Williams et al. (2007) further studied OleD and found a triple point mutant with much broader substrate specificity. The most contributing single mutation was located within Nα3. In this study, we also changed the SrUGT76G1 substrate/product spectrum or improved its catalytic efficiency through structure-based mutation on two helices (α2 and α8). We thus confirmed that α2 (corresponding to Nα3) is highly related to acceptor specificity and additionally found that the α8 helix also involved in acceptor specificity.
Figure 6. Binding of Sugar Moieties Involves S147, H155, and T284.

(A) Sugar-binding pocket observed in the complex of UDP-RebB-SrUGT76G1.
(B–F) Key residues interacting with C13-linked sugar (Glc1), C2’-linked sugar (Glc2), and C3’-linked sugar (Glc3) in SrUGT76G1 (B); in vitro enzymatic activities of H155 mutants (C); in vitro enzymatic activities of S147 mutants (D); in vitro enzymatic activities of T284 mutants toward RebA and RebD (E); manufacture of RebM using SrUGT76G1 T284S mutant (F).

From our dissolved structure of SrUGT76G1, we concluded that it is the large pocket size that confers SrUGT76G1 the ability to accommodate different sugar acceptors such as di- or monoglucosides. The present research provided more details of the recognition mechanism of diterpenoid compounds, answering why the spacious binding pocket of sugar acceptor and specific residues could affect the broad substrate spectrum of SrUGT76G1. Valuable information was obtained for answering why the spacious binding pocket of sugar acceptor and specific residues could affect the broad substrate spectrum of SrUGT76G1. Valuable information was obtained for generation and modification of the important steviol glycosides and related diterpenoid glycosides using synthetic biology approaches.

METHODOLOGIES

Plasmids, Strains, and Chemicals

E. coli DH10B was used for plasmid propagation and construction. E. coli BL21(DE3) was used for gene expression. The vector pETDuet-1 was used to express SrUGT76G1. Strains for mutated plasmid construction were grown in Luria broth (LB) and selected on 100 mg/l ampicillin. Purification of DNA fragments and extraction of plasmid DNA were performed using an AxyPrep Plasmid Miniprep Kit (Axygen Biosciences, CA, USA).

Authentic samples of steviol, stevioside, Sb, RebA, RebD, and RebM were purchased from Yuanxe Biotechnology, Shanghai, China. Rub was obtained from Guangrun Biotechnology, Nanjing, China. Sm, [l-0-D-gluco-

syl-kaur-19-enoate (K19G), steviol 19-O-glucoside (S19G), RebB, and RebI were made by our laboratory.

Cloning of SrUGT76G1 and Construction of SrUGT76G1 Mutants

The native SrUGT76G1 gene (codon-optimized in E. coli; Genscript, Nanjing) was subcloned from SrUGT76G1-cloning vector by PCR with specific primer pairs (Supplemental Table 3), digested with BamHI and HindIII, and ligated into BamHI/ HindIII-predigested vector pETDuet-1, resulting in plasmid pQZ11.

For the construction of SrUGT76G1 mutants, PCR amplification was performed with designed primers (Supplemental Table 4) using pQZ11 as template. After digestion of the template DNA with DpnI, the amplified PCR products were purified by gel electrophoresis and transformed into E. coli DH10B.

Protein Expression and Purification

Both sequencing-verified native and mutated SrUGT76G1-harboring plasmids were transformed into E. coli BL21(DE3) for protein overexpression. The transformants were selected on LB plates supplemented with ampicillin at 37°C for one night. The overnight cultures were inoculated (1:100) into 6 l of LB with ampicillin selection and grown at 37°C in an MQD-S2R shaker at 200 rpm for 2 h. When OD600 reached 1.0, a final concentration of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression. The cultures were further grown at 16°C for 18 h. The cells were harvested and resuspended in buffer A (20 mM Tris–HCl [pH 8.0] and 100 mM NaCl) supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 mM MgCl2, and 5 μg/ml DNase I. The harvested cells were crashed by a C3 high-pressure cell disruptor (Sunnybay Biotech, Canada) at 15 000 psi, and the lysate was centrifuged at 10 000 rpm for 120 min (Centrifuge 5804R, Eppendorf). The supernatant was loaded onto a Ni2+-NTA affinity column (Qiagen), incubated at 4°C for 1 h, and washed with buffer A supplemented with 25 mM imidazole. The target proteins were eluted by buffer A supplemented with 250 mM imidazole and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (30 000 molecular weight cutoff [MWCO], EMD Millipore). The protein was further purified by gel filtration using a Superdex 200 column (GE Healthcare) eluted with buffer A. Peak fractions were collected, checked by SDS-PAGE, and concentrated using an Amicon Ultra-4 Centrifugal
Structure of Glycosyltransferase SrUGT76G1

**Figure 7. Mutation of L126 and L379 Influenced the Substrate Preference.**

(A) L126 and L379F locate at the two sides of rebaudioside B.

(B) Enzyme activity of L126 and L379 mutants toward different diterpenoids. Substrate types: (I) C13/C19-O-sophorosyl substrates; (II) C13/C19-O-glucosyl substrates.

**Juncik et al., 2018.** The pocket and probe parameters were set as 4.0 and 1.4, respectively.

In Vitro Enzymatic Assay

A typical reaction (100 μl) consists of 10 μg of purified SrUGT76G1 (or SrUGT76G1 mutants) protein, 1.5 mM UDPG as sugar donor, 250 μM diterpenoid compounds as acceptors, and buffer A (20 mM Tris–HCl [pH 8.0] and 100 mM NaCl). Reaction mixtures were incubated at 37°C overnight and quenched by adding 100 μl of methanol. The reaction mixtures were centrifuged at 12 000 g for 10 min. The resulting solution was filtered through a 0.22-μm membrane and stored at 4°C for subsequent high-performance liquid chromatography (HPLC) analysis.

For the measurement of kinetic parameters of SrUGT76G1 toward different substrates, the reactions were incubated at 37°C with varying reaction times (Sb, 10 min; K19G, S19G, stevioside, and RebD, 30 min; Sm, 1.5 h; RebA, 2 h). The reaction mixtures (100 μl) consist of buffer A (20 mM Tris–HCl [pH 8.0] and 100 mM NaCl), 10 μg purified SrUGT76G1 protein, 2 mM UDPG, and sugar acceptor with a concentration varying from 10 to 2000 μM. The reaction was stopped by adding equal-volume methanol. The enzyme kinetic parameters were calculated by quantification of the formation of glycosylated products. All data were presented as means ± SD from triplicate measurements. Michaelis–Menten curves were generated by GraphPad Prism 7.

To calculate the relative conversion rate of SrUGT76G1 mutants, we used wild-type SrUGT76G1 as a control. Six diterpenoid substrates, namely Sm, Sb, Rub, stevioside, RedA, and RebD, were tested in identical conditions. The relative conversion rates (%) of SrUGT76G1 mutants were calculated as: glycosylated product amount of mutants/glycosylated product amount of native SrUGT76G1 × 100%.

**Manufacture of RebM from RebA**

The plasmid pJU651 and pHU830 used for conversion of RebA to RebM were constructed by cloning of EUGT11 (1,2-glucosyltransferase, catalyzing RebA to RebD), SrUGT76G1 (1,3-glucosyltransferase, catalyzing RebD to RebM), and AtSUS3 (A. thaliana sucrose synthase for UDPG regeneration) into pDuet-1 vector. The EUGT11 gene (codon-optimized for E. coli, Genscript, Nanjing) was subcloned from EUGT11-cloning vector by PCR with specific primer pair YF09 F and YF09 R (Supplemental Table 5) to give a fragment of AtSUS3. The PCR product was then infused into FseI and Kpnl (NEB) predigested vector pETDuet-1, resulting in plasmid pYF09. Subsequently, cDNA from A. thaliana was used as template and amplified with primer pair Ats3infuYF09-Fse and Ats3infuYF09-Kpn (Supplemental Table 5) to give a fragment of AtSUS3. The PCR product was then infused into FseI and Kpnl (NEB) predigested vector pYF09, resulting in pHU830. To construct pHJ651 and pHJ830, we amplified the vector backbone from pHU830 with primer pair 830VF and 830VR, while insert fragment of SrUGT76G1 and SrUGT76G1_T284S was amplified from plasmid pQZ11 and pQZ11_T284S with primer pair inF and inR, respectively. The amplified vector backbone and fragments were ligated using a CloneExpress II One Step Cloning Kit (Vazyme, Nanjing), resulting in plasmid pHJ651 and pHJ830.
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The overnight cultured *E. coli* BL21(DE3) harboring plasmid pHJ651 (strain 651) or plasmid 830 (strain 830) were inoculated into 10 ml of LB supplemented with 100 μg/ml ampicillin, grown at 37°C for 4 h, then inoculated into 1 L of LB (1% v/v) with ampicillin selection, and grown at 37°C at 200 rpm for 2 h. When OD_{600} reached 0.5, a final concentration of 0.1 mM IPTG was added to induce protein expression at 22°C and cultured for 20 h. Cells were harvested by centrifuge and resuspended with buffer A (100 mM PBS [pH 8.0], 60 mM sodium citrate, 40% sucrose [v/v], 1 mM ZnCl_{2}, and 5 mM ReBa) to a final OD_{600} of 100. The reaction mixture was incubated at 37°C for 48 h. The reaction mixture was centrifuged at 12 000 g for 10 min. The supernatant was filtered through a 0.22-μm membrane and stored at 4°C for subsequent HPLC analysis.

Product Detection by HPLC

The stored reaction mixture solutions or fermentation extracts were subjected to HPLC analysis (Dionex UlitMate 3000 SD HPLC system, Thermo Scientific, MA, USA). The separation was achieved on a C18 column (Sil-Green ODS column [ø 4.6 × 250 mm, S-5 μM], Greenherbs, Beijing, China) with a flow rate of 1 ml/min at 40°C. A linear gradient elution was performed with mobile phases containing acetonitrile (A) and H_{2}O (B). For detection of the product of K19G: 0 min: 40% A in 60% B; 0–10 min: linear gradient increase to 80% A in 20% B; 10–15 min: 100% A; 15–16 min: 40% A in 60% B; 16–20 min: 5% A in 95% B. For detection of the product of S19G, Sm: 0 min: 20% A in 80% B; 0–10 min: gradient increased to 60% A in 40% B; 10–15 min: 100% A; 15–16 min: 5% A in 95% B; 16–20 min: 40% A in 60% B; 20–20 min: 20% A in 80% B. For detection of the product of Sb, Rub: 0 min: 15% A in 85% B; 0–10 min: linear gradient increase to 55% A in 45% B; 10–15 min: 100% A; 15–16 min: 15% A in 85% B; 16–20 min: 15% A in 85% B. For detection of the product of steviol, RedA, RedD: 0 min: 5% A in 95% B; 0–10 min: linear gradient increase to 45% A in 55% B; 10–15 min: 100% A; 15–16 min: 5% A in 95% B; 16–20 min: 5% A in 95% B.

ACCESSION NUMBERS

The atomic coordinates and structure factors for the structures have been deposited in the Protein Data Bank with accession codes PDB: 6KVI, 6KVJ, 6KVK, and 6KVL.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Plant Communications Online.

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AUTHOR CONTRIBUTIONS

Y.W. and P.Z. conceived the study, Z.L., J.L., and Y.S. performed the experiments, Z.L., J.L., and Y.S. prepared the manuscript. Y.W. and P.Z. wrote the paper. All authors contributed to discussion of the manuscript.

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