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NOTE

Biosynthesis of ferulic acid 4-*O*-glucoside and feruloyl glucoside using *Escherichia coli* harboring regioselective glucosyltransferases

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Abstract Twelve candidate uridine diphosphate-dependent glucosyltransferases (UGTs) from *Arabidopsis thaliana* were screened to identify UGTs that synthesized ferulic acid 4-*O*-glucoside or feruloyl glucoside, respectively. We used biotransformation using *E. coli* harboring each UGT. *E. coli* harboring AtUGT71C1 could synthesize feruloyl glucoside with a conversion rate of 1.8 μM/h. *E. coli* harboring AtUGT72E2 showed the best conversion rate for converting ferulic acid into ferulic acid glucoside, at 15.8 μM/h. Molecular docking analysis of ferulic acid into the modeled structures of AtUGT71C1 and AtUGT72E2 was used to elucidate the different conversion rates of ferulic acid into corresponding glucoside.

Keywords Ferulic acid · Ferulic acid 4-*O*-glucoside · Feruloyl glucoside · Glycosyltransferase

Introduction

Glycosylation of the second metabolites occurs during the final stage of biosynthesis (Vogt and Jones 2000). The glycosylation of lipophilic compounds is carried out by a family 1 glycosyltransferase called uridine diphosphate-dependent glycosyltransferase (UGT) (Bowles et al. 2006). UGTs use uridine diphosphate (UDP) sugars as sugar donors and various compounds including hormones and secondary metabolites as sugar acceptors. UGTs show substrate specificity for sugar donors as well as sugar acceptors, but the molecular basis for the substrate specificity of UGTs remains unknown. However, some studies have shown that a certain amino acid of UGT is critical to the recognition of the sugar donor. For example, UDP-galactose:anthocyanin galactosyltransferase from *Aralia cordata* (ACGaT) gained glucosyltransferase activity after one amino acid was altered (Kubo et al. 2004). UGT78D3 from *A. thaliana*, which used UDP-arabinose as a sugar donor, preferably utilized UDP-xylose after one amino acid (His380Glu) was changed (Kim et al. 2013; Han et al. 2014). These amino acids are located in the PSPG (a plant secondary product GT consensus sequence) motif at the *C*-terminus of UGTs.

UGTs that use hydroxycinnamic acids (HCs) as a substrate have been studied in several plants. In particular, UGTs from the model plant *Arabidopsis thaliana* showed affinity for hydroxycinnamic acids, including cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid (Lim et al. 2001, 2003; Lanot et al. 2006). However, some UGTs originally identified as hydroxycinnamic acid glucosyltransferases also use flavonoids as substrates (Lim et al. 2003). The structure of HC reveals two possible glucosylation sites; the first one is at the hydroxyl group(s) and the other is at the carboxyl group. We were

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interested in determining how UGT glucosylates different glucosylation sites of HC. We screened several UGTs and found UGTs that produced ferulic acid 4-*O*-glucoside or feruloyl glucoside. Using molecular modeling, we elucidated the molecular basis of the different regioselectivities of two UGTs and their conversion rates of ferulic acid. In addition, using site-directed mutagenesis based on the molecular structure, we could alter the glucosylation pattern of ferulic acid from the carboxyl group of ferulic acid to the hydroxyl group of ferulic acid. This is the first report to demonstrate a change in a glucosylation pattern of UGT by altering one amino acid.

Materials and methods

Twelve UGTs from *A. thaliana* were cloned using reverse transcription polymerase chain reaction (supplementary Table 1). Each PCR product was subcloned into a pGEMT-easy vector (Promega, Madison, WI, USA) and sequenced. Each AtUGT was subcloned into *Escherichia coli* expression vector pGEX 5X-3. Site-directed mutagenesis was carried out using a QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, USA).

E. coli transformant was grown as described in Kim et al. (2015). For the analysis of ferulic acid 4-*O*-glucoside, the mixture was extracted with 2 volumes of ethyl acetate, and the upper phase was collected after centrifugation and dried in vacuum. The reaction product was dissolved with dimethyl sulfoxide. For the analysis of feruloyl glucoside, the reaction mixture was centrifuged, and the supernatant was collected. The supernatant was boiled for 3 min, centrifuged for 10 min, and then analyzed using high-performance liquid chromatography (HPLC). The analysis conditions were described in Kim et al. (2015). The structures of the reaction products were determined using nuclear magnetic resonance spectroscopy (NMR). The NMR data were as follows: feruloyl glucoside: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.72 (d, $J = 15.9$ Hz, 1H), 7.20 (s, 1H), 7.10 (d, $J = 8.2$ Hz, 1H), 6.82 (d, $J = 8.2$ Hz, 1H), 6.40 (d, $J = 15.9$ Hz, 1H), 5.57 (d, $J = 7.6$ Hz, 1H), 3.89 (s, 3H), 3.85 (d, $J = 13.9$ Hz, 1H), 3.69 (dd, $J = 12.1$, 4.4 Hz, 1H), 3.37–3.48 (m, 4H).

Ferulic acid 4-O-glucoside ^{1}H NMR (CD_3OD, 400 MHz) δ 7.62 (d, $J = 15.9$ Hz, 1H), 7.25 (s, 1H), 7.11–7.19 (m, 2H), 6.39 (d, $J = 16.0$ Hz, 1H), 4.97 (d, $J = 7.2$ Hz, 1H), 3.90 (s, 3H), 3.88 (d, $J = 15.0$ Hz, 1H), 3.69 (dd, $J = 11.8$, 4.7 Hz, 1H), 3.39–3.53 (m, 4H). These data were compared with previously published results (Johnsson et al. 2002; Katsuragi et al. 2011).

The three-dimensional structures of AtUGT71C1 and AtUGT72E2 were constructed using the 'First Approach Mode' on the Swiss-Model protein structure homology modeling server (http://us.expasy.org) (Han et al. 2014). The crystal structures of *Medicago truncatula* UGT71G1 (PDB ID: 2acv, 44.9 % sequence identity) and *A. thaliana* bifunctional *N*- and *O*-glucosyltransferase (PDB ID: 2vg8, 39.8 % sequence identity) were used as templates for AtUGT71C1 and AtUGT72E2, respectively.

Results and discussion

Characterization of two different glucosylated products

Ferulic acid has two possible glucosylation sites: one forms feruloyl glucoside and the other forms ferulic acid 4-*O*-glucoside (Fig. 1). In order to identify UGTs that form feruloyl glucoside or ferulic acid 4-*O*-glucoside, 12 UGTs from *A. thaliana* were tested. These 12 UGTs are known to transfer the glucose group from UDP-glucose to small compounds such as HCs (Lim et al. 2003, 2005) and monoterpenoids (geraniol and perillyl alcohol) (Caputi et al. 2008). We used biotransformation instead of in vitro enzyme assay because UDP-glucose is expensive and we did not have to purify each recombinant protein. Each transformant was fed ferulic acid because ferulic acid contains one hydroxyl group and one methoxy group. The reaction product was analyzed using HPLC. Transformant harboring AtUGT71C2, AtUGT72B1, AtUGT73C1, AtUGT76D1, AtUGT76E2, AtUGT76E12, or AtUGT85A1 did not produce any new peak, indicating that these seven AtUGTs did not glucosylate ferulic acid. Analysis of reaction product from the transformant harboring AtUGT71C1 produced a new peak at 7.2 min (Fig. 2C). The other transformant harboring AtUGT72E2, AtUGT73C3, AtUGT73C5, or AtUGT73C6 produced a peak at 6.2 min (Fig. 2B). Among these four transformants, the transformant harboring AtUGT72E2 synthesized the most product. The molecular mass of these two reaction products was 356 Da, indicating that each product had one glucose molecule attached. These results suggested that the two reaction products were glucosylated at different positions because they showed different retention times but the same molecular mass. The structure of each product was determined using NMR. The structure of the product produced by AtUGT71C1 was determined to be feruloyl glucoside, in which the glucose was attached through an ester linkage to the carboxyl group of ferulic acid. On the other hand, AtUGT72E2 glucosylated the 4-OH of ferulic acid, and the structure was determined to be ferulic acid 4-*O*-glucoside.

We compared the conversion rates of ferulic acid into the corresponding glucoside. *E. coli* harboring AtUGT71C1 converted ferulic acid into feruloyl glycoside

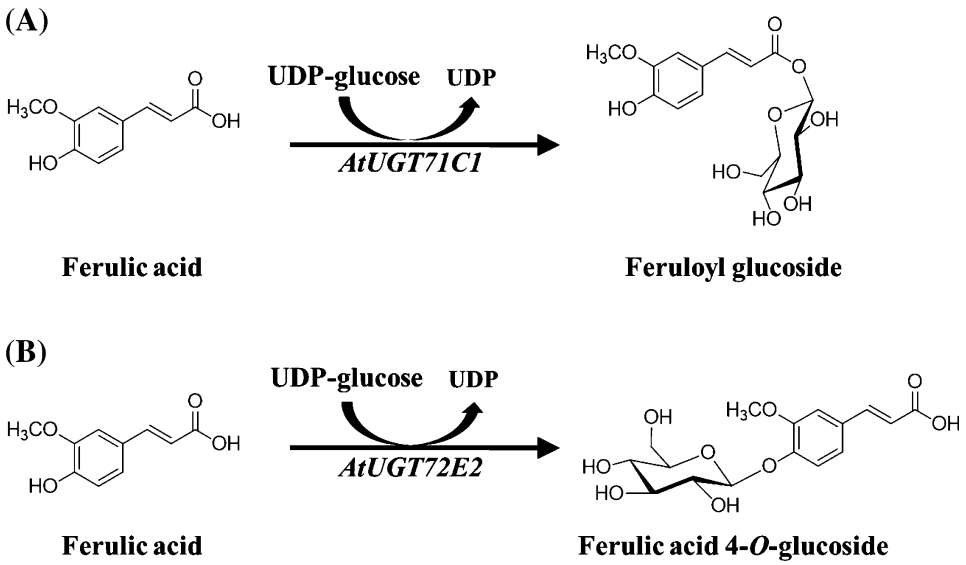

Fig. 1 Two possible glucosylation sites on ferulic acid. (**A**) Glycosylation of the carboxylic acid of ferulic acid results in the formation of feruloyl glucoside. (**B**) Glycosylation of the 4-hydroxy group of ferulic acid leads to the formation of ferulic acid 4-*O*-glucoside

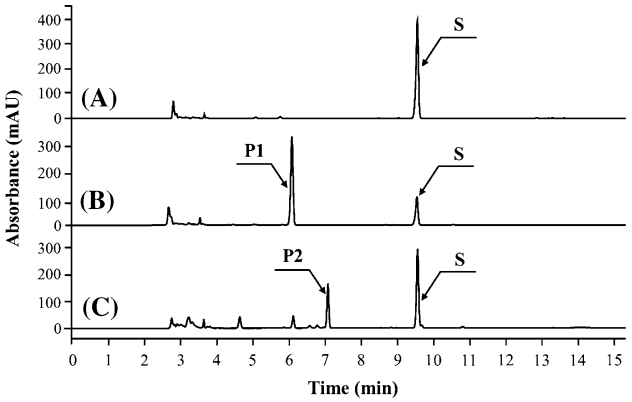

Fig. 2 Analysis of the reaction product from *E. coli* harboring UGT. (**A**) Ferulic acid standard; (**B**) biotransformation of ferulic acid using *E. coli* harboring AtUGT72E2; (**C**) biotransformation of ferulic acid using *E. coli* harboring AtUGT71C1. *S* ferulic acid, *P1* ferulic acid 4-*O*-glucoside, *P2* feruloyl glucoside

at 1.8 μM/h, while *E. coli* harboring AtUGT72E2 converted ferulic acid into ferulic acid glucoside at 15.8 μM/h. The production of ferulic acid 4-*O*-glucoside was much faster than that of feruloyl glucoside. We assumed that the different glucosylation rates of these two AtUGTs might be due to their reaction mechanisms. In order to provide a reasonable explanation for this observation, we generated the three-dimensional structures of AtUGT72E2 and AtUGT71C1 and a docking study of the substrate, ferulic acid, into the modeled structures was performed. Figures 3A and B shows the docking modes of ferulic acid to AtUGT72E2 and AtUGT71C1, respectively, which indicated a good match with the regioselectivity of the glucosylation of the corresponding enzyme. Thus, in the docking mode to AtUGT72E2, ferulic acid was positioned in such a way that the 4-OH group attacks UDP-glucose (arrow, Fig. 3A) to produce ferulic acid 4-*O*-glucoside. On the other hand, the docking mode of ferulic acid to AtUGT71C1 resulted in the preferential formation of feruloyl glucoside, where the carboxylate group of ferulic acid was docked near UDP-glucose (arrow, Fig. 3B). The binding mode analysis of ferulic acid to AtUGT72E2 revealed that the glucosyl transferase had the characteristic charge transfer system; His18 served as a base to deprotonate the phenolic hydroxyl group of ferulic acid, while Asp11 stabilized protonated His18 through an electrostatic interaction. This charge transfer system was found in other plant UGTs, and both Asp and His are common conserved residues in these UGTs. Thus, the molecular docking study showed that AtUGT72E2 would facilitate the glucosylation of ferulic acid through regiospecific binding as well as induction of deprotonation followed by stabilization of the resulting phenolate anion by the charge transfer system. In contrast, the active site of AtUGT71C1 lacked the His–Asp charge transfer system. In addition, AtUGT71C1 was suited for accommodating the carboxylate group of ferulic acid, instead of the phenolic –OH, in close proximity to UDP-glucose. As a result, the docking conformation of ferulic acid to AtUGT71C1 was not as stable as that bound to AtUGT72E2; Thr153 and Glu92 interacted with the carboxylate group and the hydroxyl group of ferulic acid, respectively (Fig. 3B). Taken together, for the glucosylation of ferulic acid by AtUGT72E2 and AtUGT71C1, the two glucosyl transferases seemed to accommodate the glucose acceptor in a distinct orientation with different degrees of stabilization, which might explain why ferulic acid 4-*O*-glucoside is produced faster than feruloyl glucoside.

According to the docking mode of AtUGT71C1, Glu92 and Thr153 of ATUGT71C1 interact with ferulic acid. It

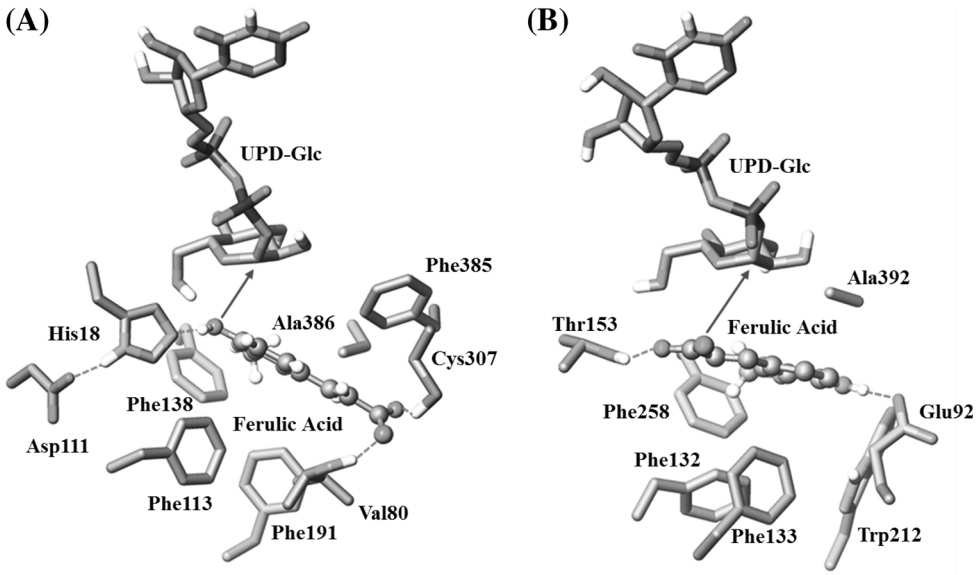

Fig. 3 Docking modes of ferulic acid to AtUGT72E2 (**A**) and AtUGT71C1 (**B**)

seemed that alteration of either amino acid resulted in changing the binding mode and producing a different product. We conducted a site-directed study in which Glu92 was changed to Ile and Thr153 was changed to Leu. The biotransformation of ferulic acid using *E. coli* harboring AtUGT71C1Glu92Ile produced a peak that had the same retention time as that of ferulic acid 4-*O*-glucoside. However, the mutation of Thr153 to Leu abolished the activity of the enzyme. Thus, the mutation of Glu92 to Ile changed the binding mode of ferulic acid, which was favorable for the glucosylation of the 4-hydroxy group of ferulic acid. This result showed that one amino acid change was able to change the regioselectivity of UGT. This is the first report showing the change of the regioselectivity of UGT by mutating one amino acid.

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