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•Research article•

Molecular cloning and functional characterization of an isoflavone glucosyltransferase from *Pueraria thomsonii*

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[ABSTRACT] *Pueraria thomsonii* has long been used in traditional Chinese medicine. Isoflavonoids are the principle pharmacologically active components, which are primarily observed as glycosyl-conjugates and accumulate in *P. thomsonii* roots. However, the molecular mechanisms underlying the glycosylation processes in (iso)flavonoid biosynthesis have not been thoroughly elucidated. In the current study, an *O*-glucosyltransferase (PtUGT8) was identified in the medicinal plant *P. thomsonii* from RNA-seq database. Biochemical assays of the recombinant PtUGT8 showed that it was able to glycosylate chalcone (isoliquiritigenin) at the 4-OH position and glycosylate isoflavones (daidzein, formononetin, and genistein) at the 7-OH or 4′-OH position, exhibiting no enzyme activity to flavonones (liquiritigenin and narigenin) *in vitro*. The identification of *PtUGT8* may provide a useful enzyme catalyst for efficient biotransformation of isoflavones and other natural products for food or pharmacological applications.

[KEY WORDS] Flavonone; Isoflavone; Glucosyltransferase; 4′-*O*-glucosylation; 7-*O*-glucosylation
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Introduction

Pueraria thomsonii roots, also known as *Fenge* in Chinese, contain abundant isoflavonoid glycosides. Puerarin, daidzin and genistein are the principle isoflavone glycosides and pharmacologically active components [1-3], and associated with numerous human health benefits, such as preventing cardiovascular diseases [4], suppressing oxidative damage induced by chronic ischemia [5] and estrogen deficiency [6]. Although these compounds exhibit important bioactivities, low water solubility is a serious drawback to their further application for the food and pharmaceutical purposes. Notably, glycosytransformation is an effective method to increase water solubility, for instance the water solubility of puerarin increased up to 14−18 times when it was glycosylated [7].

Isoflavonoids have diverse structures, which mainly depend on the functions of oxidase, isopentenyltransferase, methyltransferase and glycosyltransferase. Glycosylation, usually catalyzed by UDP-dependent glycosyltransferases (UGTs) in plants, is an important reaction in isoflavone biosynthesis [8]. However, glycosyltransferases in natural plants usually belong to the family 1 UGTs, which is characterized by the glycosyltransferase consensus sequence (PSPG) motif [9]. Both *Pueraria lobata* and *P. thomsonii* belong to *Pueraria* species of Leguminosae, and the glycosylated forms of PSPG motif were also detected in *P. lobata* tissues, suggesting the presence of various UGTs specific for glycosylation of these compounds. For example, puerarin (daidzein 8-*C*-glucoside) [10], daidzin (daidzein 7-*O*-glucoside), and genistin (genistein 7-*O*-glucoside) [11-13] all accumulated in *P. lobata* roots. Although more than 100 putative UGT genes were predicted from *P. lobata*, several UGTs were functionally characterized in *P. lobata* [10, 14-17], including *C*- and *O*-glucosytransferases, which have been molecularly characterized [15, 18]. Although *P. thomsonii* has a similar chemical composition and medicinal properties compared with *P. lobata*, the glucosytransferase in (iso)flavones metabolism, enzymes or genes that are required for these chemical modifications remain uncharacterized in *P. thomsonii*.

Previous studies identified *C*-glucosylation of daidzein for puerarin biosynthesis from *P. lobata* [10]. With the purpose of elucidating glucosylation in puerarin biosynthesis and

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identifying the corresponding UGTs, we reported the use of *P. thomsonii* RNA-seq database to identify a gene (designated *PtUGT8*) encoding a glucosytransferase responsible for puerarin biosynthesis. The biochemical properties of PtUGT8 were extensively studied *in vitro* using the crude enzyme extracts of recombinant protein obtained by heterologous expression of *PtUGT8* gene in *Escherichia coli*. The identification of *PtUGT8* will help to decipher *P. thomsonii* isoflavone tailoring process and afford the possibility of increasing water solubility to make relevant compounds suitable for food and clinical applications.

Materials and Methods

Plant materials and chemicals

P. thomsonii roots were collected from plants grown in Hengfeng County (Jiangxi Province, China). All materials were authenticated as the root of *P. thomosonii* (Willd.) Ohwi by the authors and a voucher specimen of each batch was deposited in the State Key Laboratory Breeding Base of Dao-di Herbs in China Academy of Chinese Medical Sciences. All plant materials were frozen in liquid nitrogen and then stored at −80 °C until use. Formononetin, ononin, isoliquiritigenin, isoliquiritin, liquiritigenin, liquiritin, daidzein, daidzin, puerarin, naringenin, genistein and genistin were purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China).

cDNA synthesis and cloning of PtUGT8

According to the previous literature on *P. lobata* [10], *PtUGT8* was locally blasted from *P. thomsonii* RNA-seq databases by BioEdit software. Total RNA was isolated from the roots of *P. thomsonii* using the Quick RNA Isolation Kit (Huayueyang Biotech Co., Ltd., Beijing, China). Then, using *P. thomsonii* root cDNA as a template, the open reading frames (ORFs) of *PtUGT8* were amplified with gene-specific primers (*PtUGT8*-F and *PtUGT8*-R, Table S1). The amplified product was then gel-purified, the primers were digested with the BamH I restriction site (Table S1), and the product was inserted into the expression vector pGEX-4T-1 in frame with a glutathione-*S*-transferase (GST) tag at the *N*-terminus (General Electric Co., USA) with a pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech Co., Ltd., China), yielding the construct pGEX-4T-1:: *PtUGT8*.

Sequence alignment and phylogenetic analysis

Multiple sequence alignment was implemented using DNAMAN Version 6.0. For phylogenetic analysis, the deduced amino acid sequences of *PtUGT8* were aligned with other members of the family 1 glucosyltransferases using the CLUSTAL X Version 2.0 program [19]. The phylogenetic tree was generated by the neighbor-joining method using the MEGA 7.0 program (using 1 000 bootstrap replications) [20]. The GenBank accession numbers of the sequences used in phylogenetic analysis are listed in Table S2.

Heterologous expression and affinity purification of PtUGT8

These vectors were sequenced for confirmation and then transformed into *Escherichia coli* Rosetta (DE3) strains for recombinant protein expression. Single colonies of the transgenic strains were cultured in LB medium containing 100 mg·L^{-1} ampicillin. Bacteria were grown at 37 °C to an OD_{600} value of 0.4−0.6, and recombinant PtUGT8 protein expression was induced by the addition of 1.0 mmol·L^{-1} isopropyl *β*-D-thiogalactopyranoside (IPTG). After 20 h of induction at 16 °C, the recombinant PtUGT8 was extracted according to the manufacturer's instructions. In brief, cell pellets were resuspended in a lysis buffer [0.1 mol·L^{-1} potassium phosphate (pH 8.0), 0.5 mol·L^{-1} NaCl, 1 mmol·L^{-1} EDTA, and 1 mmol·L^{-1} dithiothreitol (DTT)] and disrupted by ultrasonication on ice. After centrifugation of the cell lysates at 5 000 × *g* at 4 °C for 15 min, the crude enzyme extracts were loaded onto a column packed with GST-binding resin (TransGen Biotech Co., Ltd., China). The recombinant PtUGT8 protein was then eluted with elution buffer [0.1 mol·L^{-1} potassium phosphate (pH 8.0), and 10 mmol·L^{-1} reduced glutathione]. The purity of the recombinant protein was examined by SDS-PAGE.

Enzyme assays

Enzyme assays were performed in 100 μL of reaction mixture, containing 97 μL of crude enzyme extracts, 1 mmol·L^{-1} UDP-glucose, and 0.5 mmol·L^{-1} acceptor substrates. The acceptors used comprised one chalcone (isoliquiritigenin), two flavonones (liquiritigenin and naringenin) and three isoflavones (daidzein, formononetin, and genistein). The reactions were performed at 30 °C overnight and then stopped by the addition of 200 μL of precooled methanol. The mixture was well shaken, centrifuged at 12 000 × *g* for 10 min, and then the supernatant was filtered through a 0.22 μm filter and subsequently analyzed.

The glycosylation activity of PtUGT8 towards different substrates were preformed under the same reaction conditions with enzyme assays, and the reaction time was changed to 30 min.

UPLC and UPLC-Q/TOF-MS analysis

The reaction products were analyzed by a Waters ACQUITY UPLC I-Class system (Waters, USA), using a Waters ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) at 40 °C. Solvent A was 0.1% formic acid water, and solvent B was acetonitrile. The samples were separated with a multistep gradient from 5% to 60% B (10 min), 60%−98% B (0.5 min), 98% B (1.5 min), 98%−5% B (0.5 min), and 5% B (2.5 min) at a flow rate of 0.4 mL·min^{-1}. The detection wavelength was set at 260 nm for the detection of flavonones and isoflavones and 370 nm for chalcone and their corresponding glucosides. Liquid chromatography-mass spectrometry was performed on an Xevo G2-S Q/TOF-MS (Waters, USA). The column and analysis methods were the same with those described above for UPLC analysis. The MS data were recorded with ranges of *m*/*z* 50−1500, and other parameters were set according to a previous report [12, 21].

Results

Cloning of PtUGT8

A UGT cDNA library was constructed from the roots of *P. thomsonii*. The full-length cDNA sequence of *PtUGT8* contained an ORF of 1329 bp, which was predicted to encode a 432 amino acid UGT protein. Blast results showed that the amino acid sequence of PtUGT8 had 96.52% identity with the amino acid sequence of PlUGT43 and 82.83% identity with the amino acid sequence of GT03H24. Mean-

while, multiple alignment results showed that the deduced amino acid sequence of PtUGT8 showed a high degree of similarity to PlUGT43 and GT03H24, of which PlUGT43 functions were characterized in *P. lobata* [10]. Therefore, phylogenic tree analysis indicated *PtUGT8* is an interesting candidate gene for the glycosylation of (iso)flavonoids in *P. thomsonii* (Fig. 1). Multiple sequence alignment revealed that *PtUGT8* contained a highly conserved PSPG motif in the *C*-terminal region, which is proposed to be the glycosyl ligand binding site [9] (Fig. S1).

PtUGT8 shows OGT activity against daidzein

For the biochemical assays, recombinant PtUGT8 protein fused with a GST-tag was produced in *E. coli*. SDS-PAGE analysis of the soluble protein from IPTG-induced *E. coli* cells expressing PtUGT8 showed the expression of recombinant PtUGT8 protein (Fig. S2). The molecular mass of purified protein was approximately 74 kDa, which was consistent with the theoretically predicted molecular mass of PtUGT8 fused with the GST-tag.

PtUGT8 was screened by homologous cloning of PlUGT43, and phylogenetic tree results showed that *PtUGT8* had a closer relationship with *PlUGT43*, while the deduced amino acid sequence of PtUGT8 also shared above 96.52% sequence identity. Therefore, PtUGT8 may have the same activity as PlUGT43. To verify this hypothesis, the crude enzyme extracts of PtUGT8 were tested for the activity with daidzein, which is the most plausible substrate for puerarin biosynthesis [10]. Control reactions were performed without the presence of recombinant proteins. Apparently, a main product (peak 1) corresponding to daidzin was produced during *in vitro* reaction with PtUGT8 (Fig. 2A). The retention time and molecular ion of peak 1 were essentially identical to those of daidzin standard (Fig. 2C). At the same time, a by-product was formed with a small amount (data not shown) with a molecular ion of *m/z* 417.11 $[M + H]^+$; it might be a daidzein monoglucoside, where a glucose group was attached to the 4′-OH position. Unfortunately, the biochemical assay data suggested that PtUGT8 possessed *O*-glycosylation activity towards daidzein. In other words, PtUGT8 is an *O*-glucosyltransferase, and does not show *C*-glycosylation activity as exhibited by PlUGT43 in a previous study, which can perform 8-*C* glycosylation of daidzein.

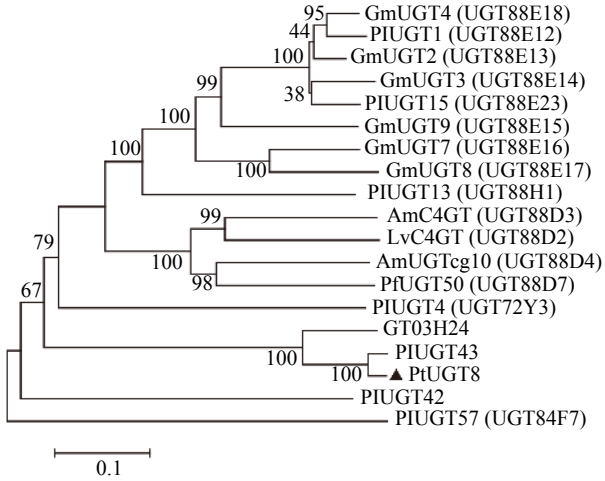

Fig. 1 Phylogenetic tree analysis of *PtUGT8* with UGTs in other plants

PtUGT8 exhibits substrate broadness for chalcone and isoflavones

Using UDP-glucose as the glucosyl donor, the glucosyl acceptor specificity of PtUGT8 was tested against a range of plausible substrates that are present in *P. thomsonii* or available commercially (Fig. 3). The results of the biochemical assays and UPLC-Q/TOF-MS analysis showed that, except for naringenin and liquiritigenin, PtUGT8 was active with the compounds tested, including chalcone (isoliquiritigenin), and isoflavones (formononetin and genistein) (Table 1).

For chalcone, PtUGT8 converted isoliquiritigenin to one product eluted in peak 3 in comparison with the control reaction (Fig. 4A). By examining the retention times and mass spectra in comparison with chemical standard, the product was identified as isoliquiritin (isoliquiritigenin 4-*O*-glucoside) (Fig. 4A, Figs. S3C−D). Peak 3 had a molecular ion of *m/z* 419.1342 $[M + H]^+$ (Fig. S3C), indicating that peak 3 might be isoliquiritigenin monoglucoside, where a glucose group was attached to the 4-*O* position. In other words, PtUGT8 is a glycosyltransferase that can catalyze 4-*O*-glucosylation activity towards isoliquiritigenin.

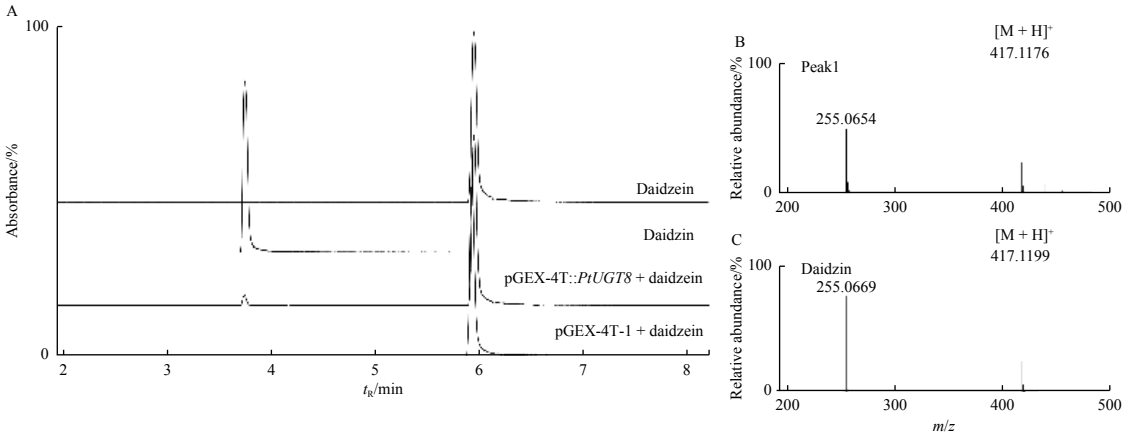

Fig. 2 A. UPLC analysis products of *PtUGT8* with daidzein and standards (daidzin, daodzein); B. The *m/z* result showed that the ion of the peak 1; C.The *m/z* result showed that the ion of the daidzin standard

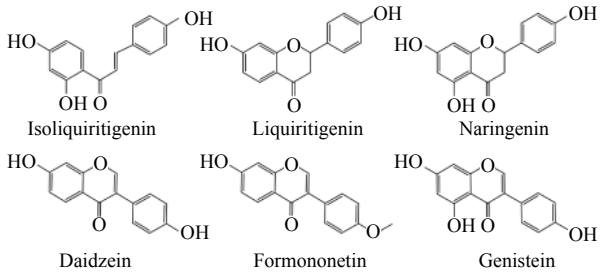

Fig. 3 Chemical structures of the substrates

The control reaction produced peak 5, expect for the substrate peak of isoliquiritigenin, which was identified as liquiritigenin based on the retention times and mass spectra in comparison with the chemical standard (Fig. 4A, Fig. S3G). Interestingly, peak 4 was also detected during *in vitro* reaction with PtUGT8 and identified as liquiritigenin (Fig. 4A, Fig. S3G). Comparison with the control reaction also identified peak 2 when PtUGT8 was incubated with isoliquiritigenin (Fig. 4A). According to the retention times and MS spectra in comparison with chemical standards, peak 2 was identified as liquiritin with a molecular ion of *m*/*z* 441.1155 $[M + Na]^+$ (Fig. 4A, Fig. S3A).

Table 1 The specific activities of PtUGT8 towards substrates

	Substrates	Product 1	Product 2
Chalcone	Isoliquiritigenin	Isoliquiritin	–
Flavonone	Liquiritigenin	–	–
	Naringenin	–	–
Isoflavone	Formononetin	Ononin	–
	Daidzein	Daidzin	Daidzein 4′-*O*-glucoside
	Genistein	Genistin	Genistein 4′-*O*-glucoside

*PtUGT8 was active with chalcone (isoliquiritigenin) and isoflavones (formononetin, daidzein and genistein), whereas it did not exert activity with any of the flavonones (liquiritigenin and naringenin). –, not applicable

When formononetin was used as the substrate, a product (peak 6) corresponding to ononin was produced in the reaction with PtUGT8 but not in the control reaction (Fig. 4B). The retention time and mass spectra of peak 6 was essentially identical to those of ononin standard (Fig. 4B, Fig. S3H). Peak 6 had a molecular ion of *m*/*z* 431.1339 $[M + H]^+$ (Fig. S3H), indicating that peak 6 might be formononetin monoglucoside, where a glucose group was attached to the 7-OH position. Therefore, these data suggested that PtUGT8

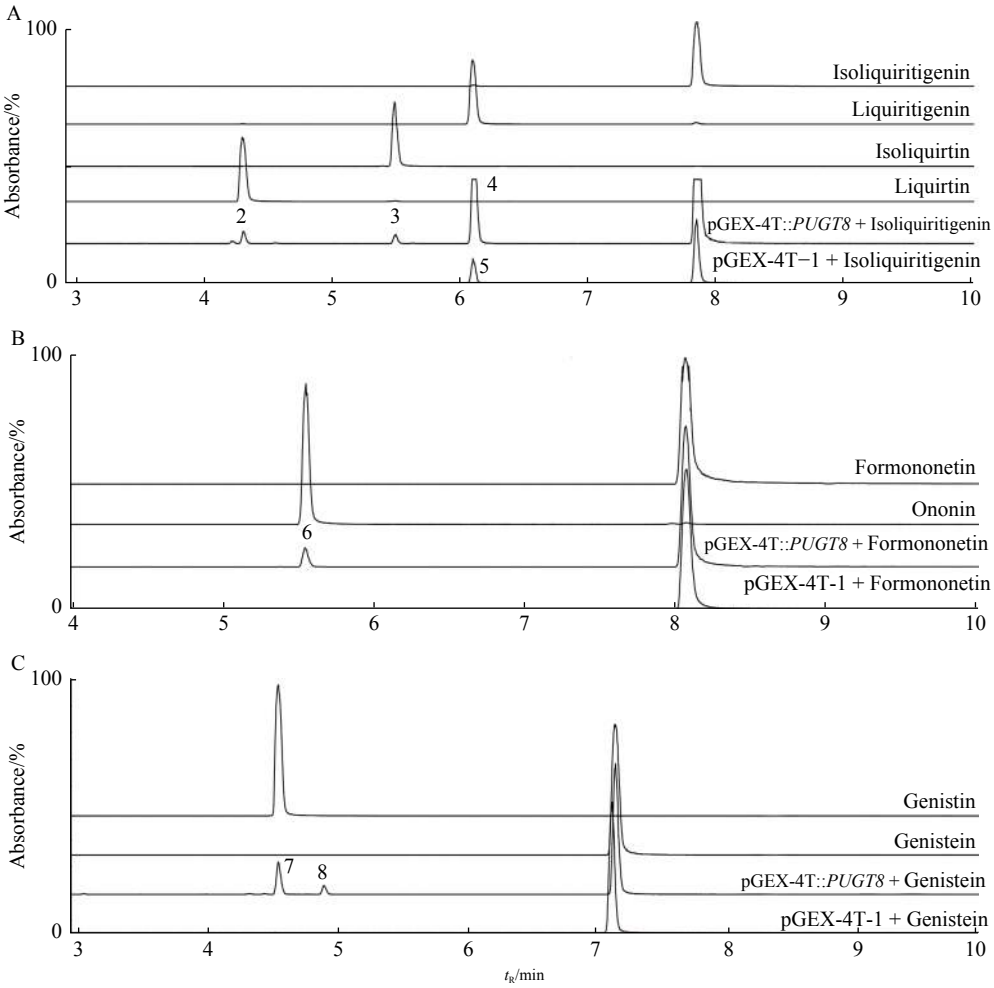

Fig. 4 UPLC analysis of the products from the *in vitro* reactions of the recombinant PtUGT8 with isoliquiritigenin, formononetin and genistein

catalyzes 7-*O*-glycosylation activity towards formononetin.

PtUGT8 transferred an isoflavone substrate (genistein) to two glycoside products eluted at peak 7 and peak 8 in comparison with the control reaction (Fig. 4C). By examining the retention times and mass spectra in comparison with chemical standards, peak 7 was identified as genistin (genistein 7-*O*-glucoside) (Figs. S3J−K), and peak 8 was identified as genistein 4′-*O*-glucoside (Fig. S3L). These data suggested that PtUGT8 catalyzes either 7-OH or 4′-OH glucosylation activity towards genistein.

In contrast to the control reaction results, no product was detected with PtUGT8 when liquiritigenin and naringenin, which are flavonones, were used as the substrates (Fig. S4A). However, peak 9 and peak 10 were also detected during *in vitro* reaction with PtUGT8 and in the control reaction, which were identified as isoliquiritigenin based on the retention times and mass spectra in comparison with the chemical standard, demonstrating similar results as those for PtUGT8 with isoliquiritigenin. No *C*-glucoside product was detected in the reactions of PtUGT8 with any of the above substrates by UPLC-Q/TOF-MS, suggesting that the *C*-glycosylation activity of PtUGT8 needs further experiments to verify due to the detection limit of the DAD detector. These results indicated that PtUGT8 possesses substrate broadness for chalcone and isoflavones tested in this study, but the glycosylation activity of PtUGT8 towards the two flavonoes (liquiritigenin and naringenin) is not detected.

Comparison of glycosylation activity of PtUGT8 towards different substrates

In order to compare the glycosylation transfer activity between different substrates, the ranges of the substrate conversion rate by the recombinant PtUGT8 were systematically studied. Four substrates were selected by *in vitro* reaction with PtUGT8 for main product conversion rate detected by UPLC, including chalcone (isoliquiritigenin), and isoflavones (formononetin, genistein, and daidzein). The *in vitro* reaction of each substrate with PtUGT8 were performed in triplicate. Among the substrates, the main product of genistein had the highest conversion rate (66.89%), followed by formononetin (52.64%) and daidzein (40.21%), and isoliquiritigenin (0.25%) (Fig.5). The results indicated that the glycosylation activity of PtUGT8 to isoflavones was higher than chalcone, and the glycosylation activity of genistein was the highest.

Discussion

P. thomsonii roots are useful in the treatment of diabetes, hyperlipidemia and cardiovascular diseases [22-23]. Isoflavones, including puerarin, daidzin and genistein, are believed to be the major bioactive components that contribute to pharmacological actions. However, these isoflavones are very hydrophobic, and their bioavailability is quite low, thus limiting their further application for clinical trials. Glycosylation can increase the bioavailability of natural drugs by rendering them more water-soluble and less toxic, and therefore molecular characterization of glycosylation has attracted increasing scientific interest.

Multiple activities of a UGT towards an acceptor at different hydroxyl groups were previously observed for *P. lobata* UGT with daidzein and genistein [16]. The present study investigated the biochemical activities of PtUGT8 towards a diversity of substrates including chalcone, flavonones and isoflavones (Fig. 3). Heterologous expression in *E. coli* cells showed that PtUGT8 not only exhibited enzyme activity to glucosylate chalcone substrate at the 4-OH position but also to glucosylate isoflavone at 7-OH or 4′-OH (Fig. 4). When daidzein or genistein were used as a substrate during *in vitro* reaction with PtUGT8, the peak areas of main products (daidzin or genistin) and by-products (daidzein 4′-*O*-glucoside or sophoricoside) were quite different due to a glucose group attached to the 7-OH or 4′-OH position, respectively. Therefore, when isoflavone was used as the substrate, PtUGT8 tended to preferentially catalyze glycosylation at the 7-OH position. The specific mechanism needs further experiments to prove whether it is caused by structure specific characteristics or others. Similarly, PtUGT8 efficiently catalyzed 7-*O*-glucosylation with formononetin, yielding ononin (Fig. 4), which indicated that methyl attachment does not interfere with glucosylation. The result of glycosylation activity comparison of PtUGT8 towards different substrates showed that the glycosylation activity of PtUGT8 to isoflavones was higher than chalcone, and genistein was the highest.

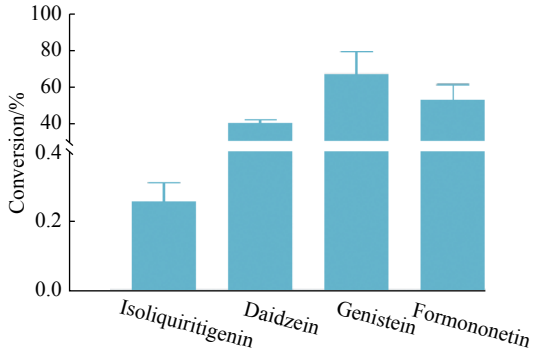

Fig. 5 Conversion rate of main products catalyzed by PtUGT8 during *in vitro* reaction

PtUGT8 selected chalcone and isoflavones as specific substrates, whereas flavonones exhibited no enzyme activity. Based on the structural formula of isoflavones and flavonones, the differences between them lie not only in the double bond and single bond at positions 2 and 3, but also in the connection position of B ring of benzene ring in their mother nucleus. Thus, we can assume that, the double bond between positions 2 and 3 in isoflavones is the key functional group of the substrate and protein. When the bond becomes a single bond, the interaction disappears, or the spatial structure greatly changes, which affects the binding of the substrate to the active site. As a result, PtUGT8 lost the ability to glycosylate flavonones.

Puerarin is an isoflavone *C*-glucoside (daidzein 8-*C*-glucoside), and a previous study identified PlUGT43 as an enzyme responsible for the *C*-glucosylation step during its biosynthesis [10]. The early metabolic steps as far as daidzein are conserved in nearly all legume species investigated, whereas puerarin only accumulates in pueraria species, indicative of a biochemically unique *C*-glucosyltransferase in the genus. It is possible that PtUGT8 is adjacent to PlUGT43,

which is able to glycosylate isoflavones at 8-*C* according to phylogenetic tree analysis [10]. Therefore, PtUGT8 was expected to be a potential candidate for our purposes in this study. Despite the closer relationship with PlUGT43 (Fig. 1), PtUGT8 does not show the same activity as PlUGT43, which specifically glucosylates daidzein at the 8-*C* position, providing an example that the biochemical function of UGTs can not be predicted simply by their primary sequences. Thus, PtUGT8 likely has been recruited from *O*-glucosyltransferase activity specific for chalcone and isoflavones.

Interestingly, in the biochemical assay with isoliquiritigenin as the substrate, we not only identified the product (isoliquiritin) but also observed the formation of liquiritigenin and its glycosides. We suspected that this finding represented the isomerization of isoliquiritigenin to produce liquiritigenin and isoliquiritigenin by glycosylation, respectively. However, the biochemical assay with liquiritigenin as the substrate showed that PtUGT8 did not catalyze liquiritigenin to liquiritin. Thus, we make a bold assumption that isoliquiritigenin and isoliquiritin are isomerized to produce liquiritigenin and liquiritin *in vitro*. In plants, we know that isoliquiritigenin can generate liquiritigenin under the catalysis of chalcone isomerase (HID). According to the results mentioned above, there is a similar biochemical reaction in the original plant, where isoliquiritigenin and isoliquiritin can spontaneously generate liquiritigenin and liquiritin without enzyme catalysis.

Conclusions

In conclusion, an *O*-glucosyltransferase PtUGT8 from the medicinal plant *P. thomsonii* was cloned and characterized. Biochemical assays in conjunction with *in vitro* data revealed its involvement in the biosynthesis of chalcone and isoflavones. Moreover, the enzyme also exhibited catalytic promiscuity in the glucosylation of different hydroxyl groups on chalcones and isoflavones, present in *Pueraria* species. Because of its broad substrate specificity, this recombinant enzyme promises to be an attractive choice for the engineering of flavonoid diversity.

Supplementary Material

Supplementray information can be acquired by e-mail to corresponding author.

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