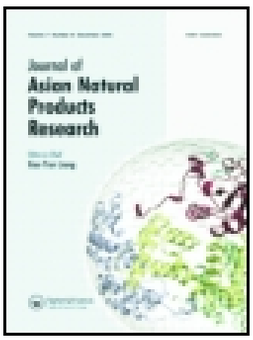

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Identification of a flavonoid 7-*O*-glucosyltransferase from *Andrographis paniculata*

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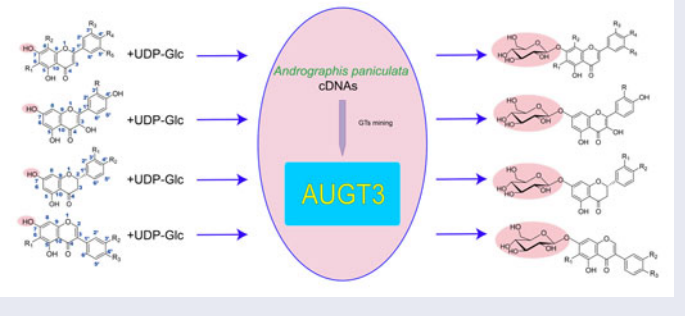
ABSTRACT

Andrographis paniculata is an important traditional medicinal herb in which flavonoids are part of the primary specialized metabolites. A flavonoid glucosyltransferase with broad substrate spectrum (named ApUGT3) was successfully identified by screening homologous glycosyltransferase genes from *A. paniculata*. The enzyme displayed glycosylation activity toward multiple flavonoids *in vitro*, and the major products were identified as 7-*O*-glucosides. Phylogenetic analysis revealed that ApUGT3 is the first reported glycosyltransferase from the Acanthaceae family that belongs to cluster I, suggesting that ApUGT3 is a new flavonoid glycosyltransferase of this subcluster. This enzyme is potentially useful as powerful glycosylation catalysts to modify flavonoid-like compounds and improve their biological activities.



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7-O-glucosyltransferase;
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1. Introduction

The medicinal plant *Andrographis paniculata* from the Acanthaceae family, known as Chuanxinlian in Chinese, has been widely used to treat fever, diarrhea, upper respiratory

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tract infections, active ulcerative colitis, and other disorders [1–4]. It has already been listed as a dietary supplement in the United States Pharmacopoeia. Flavonoids and their glycosides are some of the main specialized metabolites in *A. paniculata* [5–7]. The flavonoids in *A. paniculata* have similar structural backbones, which include two phenyl groups and a heterocyclic ring connected by a fatty chain or oxygen heterocycles [8]. As shown in recent studies, the administration of a combination of quercetin and dasatinib removes senescent cells from mouse tissues, restores physiological functions, and prolongs the life span of aging mice [9]. These findings were also confirmed in humans [10]. However, the chemical synthesis of flavonoid glycosides is restricted by the introduction of protective groups, the requirement for toxic and explosive catalysts, low yield, and side effects [11–12].

Glycosylation is one of the frequently occurring modifications in nature that yields natural products with promising applications in new drug development. The enzymes that catalyze glycosylation reactions are members of the glycosyltransferase (GT) superfamily [13]. GTs are important because they confer pharmacological properties to the compound, leading substantial changes in solubility, stability, toxicity or bioactivity, and improved pharmacokinetic parameters of metabolites [14–16]. GTs are encoded by a multi-gene family, accounting for approximately 1%–2% genes in higher plants, making the characterization of the target genes that are involved in the biosynthesis of specific glycosylated compounds difficult and complex. Transcriptome sequencing technologies are powerful tools to initially screen new genes. In our previous study, nine GTs were screened from the transcriptome databases of *A. paniculata* [17]. However, the functions of most UGTs from *A. paniculata* have not been characterized.

In this study, a UGT gene, isolated by transcriptome sequencing from the leaves of *Andrographis paniculata* (ApUGT3), was cloned, heterologously expressed, and its recombinant enzyme was purified from *E. coli*. An analysis of the substrate spectrum revealed that ApUGT3 exhibited substrate promiscuity and used flavonoid compounds as substrates. The enzyme is potentially useful for modifying flavonoid-like compounds to improve their biological activities and for synthetic biology.

2. Results and discussion

2.1. Isolation and identification of ApUGT3

The cDNA sequence of ApUGT3 (1365 bp, GenBank accession number: MH379335) was deduced to encode a 454-amino acid protein, with a relative molecular weight (MW) of 49.246 kDa and isoelectric point (pI) of 6.04. A BLASTP search was performed to identify homologues of the ApUGT3 gene. Amino acid sequences of ApUGT3 and other corresponding sequences downloaded from GenBank were aligned using DNAMAN software. ApUGT3 showed the highest identity (63.47%) with a UDP-glucuronosyltransferase and a UDP-glucosyltransferase from *Handroanthus impetiginosus*. ApUGT3 also shared 60.98% identity with a flavonoid glucosyltransferase from *Perilla frutescens*, and 60.88% identity to a flavonoid glycosyltransferase from *Scutellaria baicalensis*. The multiple sequence alignment revealed the conservation of

the plant secondary product glycosyltransferase motif (PSPG) in ApUGT3 and other plant UGTs.

2.2. Phylogenetic analysis

A neighbor-joining phylogenetic tree constructed based on the putative ApUGT3 protein and other reported flavonoid glycosyltransferases revealed that ApUGT3 was grouped into cluster I (Figure 1). The enzymes in this cluster are capable of catalyzing the glycosylation of flavonoid compounds with various structures, such as flavonols and anthocyanidin substrates. [18]. Based on the results, ApUGT3 was assigned as a putative glycosyltransferase capable of catalyzing the glycosylation of flavonoid compounds with various structures.

2.3. Heterologous expression and product identification

Twenty-one structurally diverse flavonoid compounds were selected as substrates, including flavones (**1**–**6**), flavonols (**7** and **8**), flavanones (**9**–**11**), isoflavones (**12**–**20**), and a dihydrochalcone (**21**) with UDPG as the sugar donor *in vitro* to explore the promiscuous catalytic activity of ApUGT3 in flavonoid glycosylation (Figure 2). Glycoside productions were verified using UPLC-PDA and UPLC-Q-TOF-MS analyses. ApUGT3 exhibited a rather high conversion rate when incubated with flavonols and produced one predominant glycosylated product and multiple minor hydroxyl substitutions on various types of flavonoids. The major products were identified as 7-*O*-glucosides by comparing the retention time (t), UV spectra (λmax), and parent ions ($[M\text{-}H]^-$) of the glycosylated products with the corresponding authentic standards (Figure 3) and the published data [19]. For some flavones and flavonols (**1**, **3**, and **8**), trace amount of di-glucosides were also detected. In addition, ApUGT3 was sufficiently flexible enough to glycosylate the C-7 hydroxyl group of flavonoids with substituents at C5, C6, C8, C3', C4', and C5' at the A ring or the B ring. The conversion rate also varied with the position of hydroxyl substitutions. Moreover, ApUGT3 displayed the greatest activity toward flavonols. Upon removal of the 3-hydroxyl group, the enzyme exhibited a relatively low activity.

3. Experimental

3.1. Chemicals and plant materials

Methanol and acetonitrile (HPLC grade) were purchased from Fisher Scientific Co. (Loughborough, UK). Formic acid (HPLC grade) and UDP-glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard flavonoid compounds and their glucosides were purchased from J & K Scientific Ltd. (Beijing, China), Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China), and BioBioPha (Kunming, China). Newly harvested seeds of *A. paniculata* were collected from Zhangzhou, Fujian Province, China. Plants were grown in a controlled environmental chamber with a constant temperature of $25 \pm 2\,^{\circ}C$ under 16-h light:8-h dark photoperiod. The leaves of four-week-old hydroponic seedlings were treated with methyl jasmonate (50 μM) for 48 h.

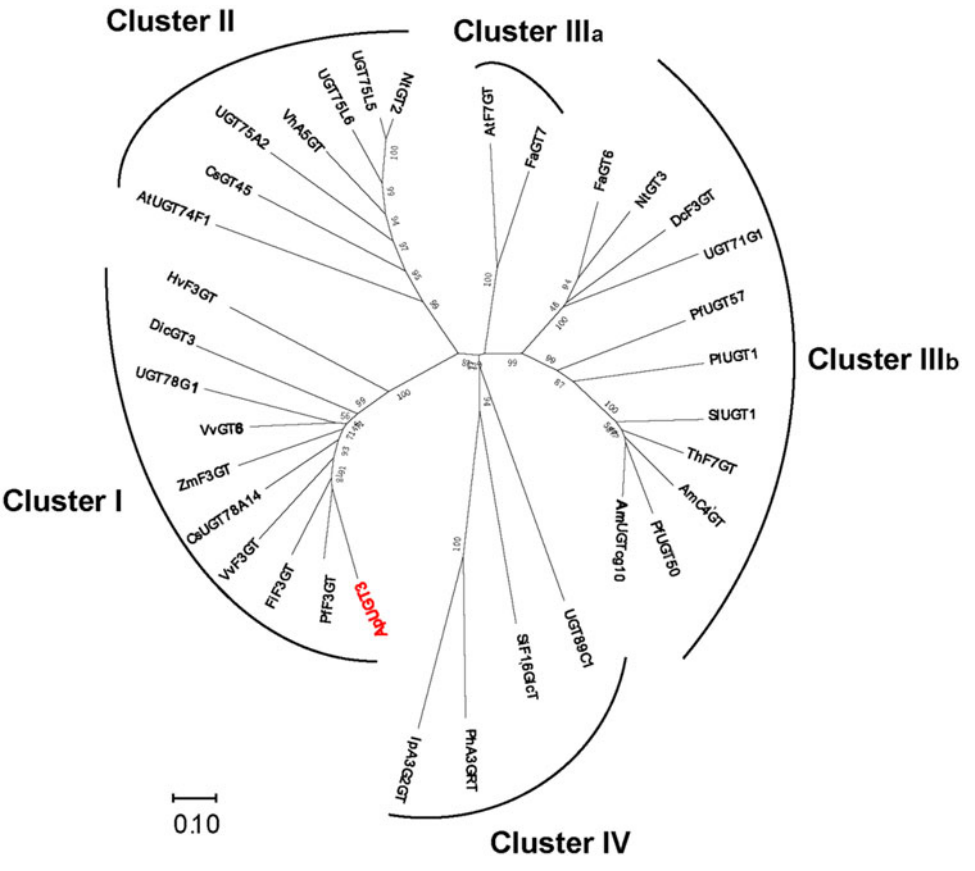

Figure 1. Phylogenetic tree of ApUFGTs and other plant UGTs. The tree was constructed using MEGA 7.0 software with a 1000 bootstrap value. Clusters (I, II, III_a, III_b, and IV) are shown in boldface letters. ApUGT3 is shown in red letters. The GenBank accession numbers and sources for the respective protein sequences are as follows: PfF3GT (BAA19659) from *Perilla frutescens*, FiF3GT (AAD21086) from *Forsythia intermedia*, VvF3GT (AAB81682) from *Vitis vinifera*, CsUGT78A14 (ALO19888) from *Camellia sinensis*, ZmF3GT(X13502) from *Zea mays*, V_VGT6 (NM_001280903) from *Vitis vinifera*, UGT78G1 (XP_003610163) from *Medicago truncatula*, DicGT3 (BAD52005) from *Dianthus caryophyllus*, HvF3GT (CAA33729) from *Hordeum vulgare*, AtUGT74F1 (NP973682) from *Arabidopsis thaliana*, CsGT45 (FJ194947) from *Crocus sativus*, UGT75A2 (AB360613) from *Lycium barbarum*, VhA5GT (BAA36423) from *Verbena hybrida*, UGT75L6 (AB555731) from *Gardenia jasminoides*, UGT75L5 (AB360620) from *Lycium barbarum*, NtGT2 (BAB88935) from *Nicotiana tabacum*, AtF7GT (AAL90934) from *Arabidopsis thaliana*, FaGT7 (Q2V6J9) from *Fragaria ananassa*, FaGT6 (Q2V6K0) from *Fragaria ananassa*, NtGT3 (BAB88934) from *Nicotiana tabacum*, DcF3GT (BAD52004) from *Dianthus caryophyllus*, UGT71G1 (AAW56092) from *Medicago truncatula*, PfUGT57 (BAG31949) from *Perilla frutescens*, PlUGT1 (A0A067YB04) from *Pueraria lobata*, SlUGT1 (AB362989) from *Scutellaria laeteviolacea*, ThF7GT (BAH14961) from *Torenia hybrid cultivar*, PfUGT50 (AB362991) from *Perilla frutescens*, AmC4′GT (AB198665) from *Antirrhinum majus*, AmUGTcg10 (AB362988) from *Antirrhinum majus*, UGT89C1 (AAP31923) from *Arabidopsis thaliana*, SiF1,6GlcT (AB333799) from *Sesamum indicum*, PhA3GRT (CAA50376) from *Petunia x hybrida*, and IpA3G2GT (BAD95882) from *Ipomoea purpurea*.

3.2. RNA isolation, cDNA synthesis, and gene cloning

Total RNA was isolated (Thermo Fisher Scientific, California, USA), and the quality was detected using a Nanodrop2000 spectrophotometer (Thermo Fisher Scientific,

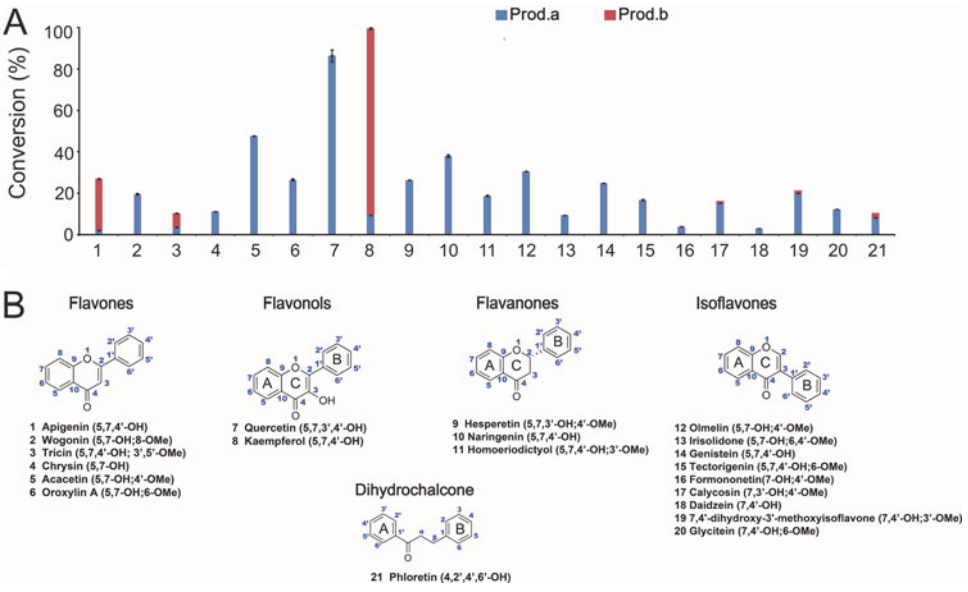

Figure 2. Exploring the promiscuous catalytic activity of recombinant ApUGT3. (A) Percent conversion of glycosylated products catalyzed by ApUGT3. The colors in the bar graphs (Prod. a and Prod. b) represent different ratios of diverse glycosylated products in the total product yield of each compound. Error bars in the figure indicate ± SDs. (B) Structures of the library members and corresponding glycosylated products.

California, USA). The first-strand cDNAs were synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) and then served as templates for PCR. The primers used in this study were ApUGT3-F (5′-TCCAGGGGCCCGAATTCGGAATGGGTTACCACTCCCACATC -3') and ApUGT3-R (5′- AGTGCGGCCGCAAGCTTGTCATTTGGAAATGCCAATTATT -3'). The PCR products were purified and cloned into a N-terminal MBP fusion expression vector HIS-MBP-pET28a (provided by Dr. Xiaohong Zhang (Karmanos Cancer Institute, United States); HIS, histidine; MBP, maltose-binding protein) using a pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China), then transformed into Trans1 T1 phage-resistant chemically competent cells (TransGen Biotech), and cultured in Luria–Bertani (LB) medium at 37 °C in the dark. Positive clones were sequenced.

3.3. Sequence analysis

Amino acid sequences for previously identified UGTs were obtained from the NCBI database, and the phylogenetic tree was constructed with MEGA7.0 software using the neighbor-joining method [20]. Branch support was estimated using a bootstrap analysis with 1000 replicates.

3.4. Expression and purification of recombinant ApUGT3

The expression plasmid was transformed into *Escherichia co*li Transetta (DE3) expressing competent cells (TransGen Biotech). The monoclonal colonies were

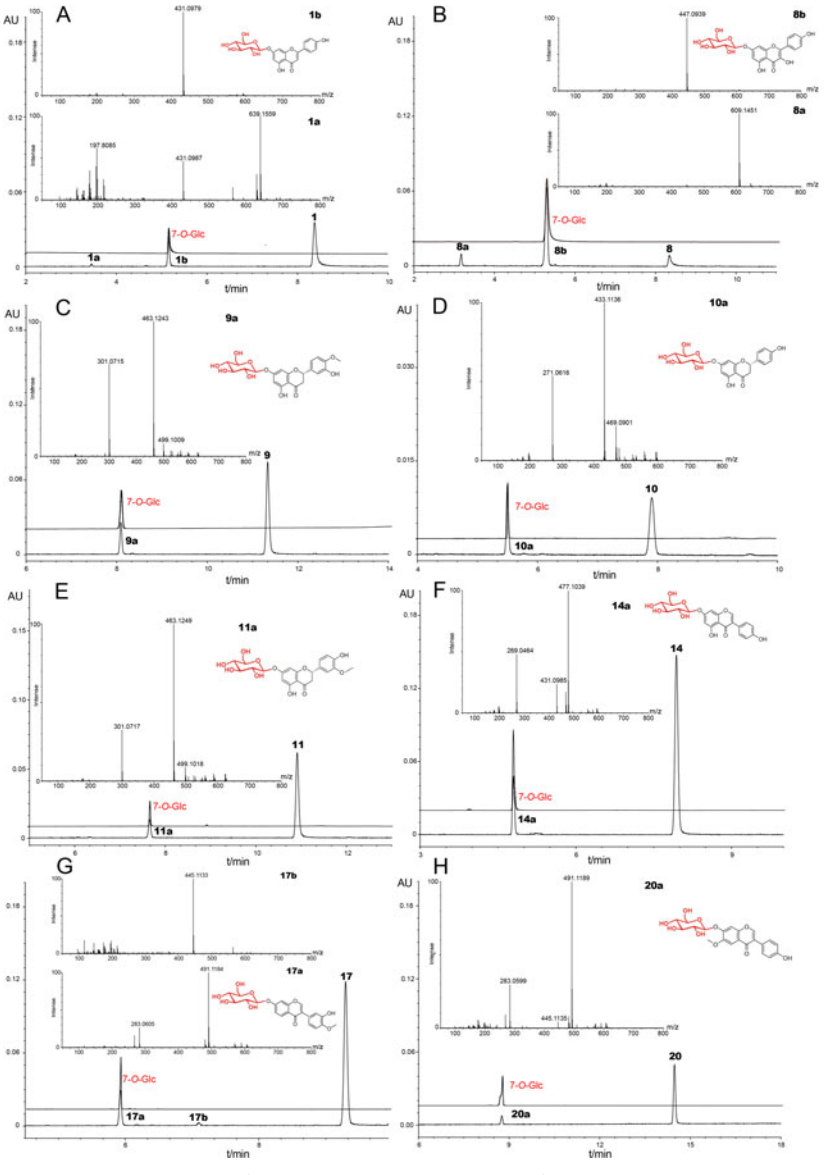

Figure 3. *In vitro* enzyme assay for determining the activity of ApUGT3 using apigenin (**1**), kaempferol (**8**), hesperetin (**9**), naringenin (**10**), homoeriodictyol (**11**), genistein (**14**), calycosin (**17**), and glycitein (**20**) as substrates. (A) UPLC chromatogram of **1** and enzyme-catalyzed products **1a** and **1b**. Insertions indicate the typical negative ion mass spectra of **1a** and **1b**. (B) UPLC chromatogram of **8** and enzyme-catalyzed products **8a** and **8b**. Insertions indicate the typical negative ion mass spectra of **8a** and **8b**. (C) UPLC chromatogram of **9** and enzyme-catalyzed product **9a**. Insertions indicate the typical negative ion mass spectra of **9a**. (D) UPLC chromatogram of **10** and enzyme-catalyzed product **10a**. Insertions indicate the typical negative ion mass spectra of **10a**. (E) UPLC chromatogram of **11** and enzyme-catalyzed product **11a**. Insertions indicate the typical negative ion mass spectra of **11a**. (F) UPLC chromatogram of **14** and enzyme-catalyzed product **14a**. Insertions indicate the typical negative ion mass spectra of **14a**. (G) UPLC chromatogram of **17** and enzyme-catalyzed product **17a**. Insertions indicate the typical negative ion mass spectra of **17a**. (H) UPLC chromatogram of **20** and enzyme-catalyzed product **20a**. Insertions indicate the typical negative ion mass spectra of **20a**.

Table 1. UPLC methods used in this study.

Method	Solvent A	Solvent B	Flow rate	Gradient	Analysis of substrates
A	0.1% formic acid	CH_3CN	0.4 mL/min	95%–83% A (0–3 min), 83%–65% A (3–12 min), 65%––40% A (12–14.5 min)	**1–3**, **7**, **9**, **11**, **17**
B	0.1% formic acid	CH_3CN	0.4 mL/min	95%–75% A (0–6 min), 75%–60% A (6–15 min)	**4–6**, **8**, **10**, **14**, **18**, **21**
C	0.1% formic acid	CH_3CN	0.4 mL/min	95%–83% A (0–10 min), 83%–65% A (10–20 min)	**12**, **13**, **15**, **16**, **19**, **20**

identified and transferred to Luria–Bertani (LB) media for heterologous expression induced by isopropyl β-D-thiogalactoside (IPTG) using previously described methods [17].

3.5. Enzyme assay and product identification

All assays and incubations were performed in 100 μl of 50 mM Tris-HCl (pH = 8.0) containing 8 μg of purified proteins, 200 μM aglycone, and 3200 μM UDP-glucose. The reactions were incubated at 30 °C for 12 h and terminated by the addition of 200 μl of methanol. The products of the reactions were filtered through a 0.22-μm nylon syringe filter and analyzed using a Waters Acquity UPLC-I-Class system (Waters Corp., Milford, MA) with an Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 50 mm). The column temperature was set to 40 °C, and the flow rate was 400 μL/min. Mobile phase A was a 0.1% formic acid aqueous solution, and mobile phase B was acetonitrile. Gradient programs were used to analyze the reaction mixtures (Table 1). The total conversion rate was calculated to be one percent of the sum of the peak areas of the substrate and product(s). The experiment was performed in the ESI (–) mode as previously described [17].

Disclosure statement

No potential conflict of interest was reported by the authors.

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