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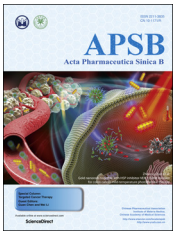

ORIGINAL ARTICLE

Biocatalytic access to diverse prenylflavonoids by combining a regiospecific *C*-prenyltransferase and a stereospecific chalcone isomerase

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Abstract Prenylflavonoids are valuable natural products that have diverse biological properties, and are usually generated biologically by multiple metabolic enzymes in nature. In this study, structurally diverse prenylflavonoids were conveniently synthesized by enzymatic catalysis by combining GuILDT, a regiospecific chalcone prenyltransferase, and GuCHI, a stereospecific chalcone isomerase that has promiscuous activity for both chalcones and prenylchalcones as substrates. Our findings provided a new approach for the synthesis of natural/unnatural bioactive prenylflavonoids, including prenylchalcones and optical prenylflavanones with chalcone origins.

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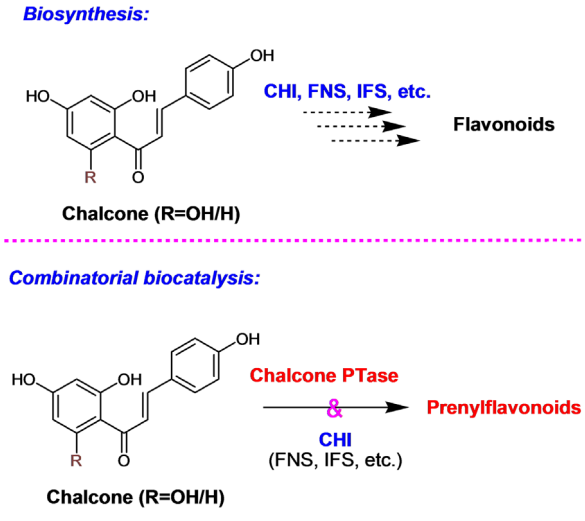
1. Introduction

Prenylflavonoids are a diverse class of naturally occurring flavonoids that contain prenyl substituents. In higher plants, these compounds usually exhibit strong antibacterial and antifungal activities[1]. Moreover, many of them have attracted attention due to their diverse pharmacological activities, such as anti-cancer, anti-obesity, anti-inflammation activity, anti-bacterial pathogens, and the impairment of autophagosome maturation[2–8]. *C*-Prenylation of flavonoids by flavonoid prenyltransferase (PTase) represents a Friedel–Crafts alkylation of the flavonoid skeleton in the biosynthesis of natural prenylflavonoids and plays a crucial role in generating the diverse compounds due to the different prenylation positions and various types of prenyl groups as well as further tailoring modifications (*e.g.*, hydroxylation and cyclization), leading to the production of more than 1000 prenylflavonoids in plants[9]. Substitutions with prenyls of different modes on the flavonoid scaffold not only contribute significantly to the structural diversity, but also markedly enhance the bioactivity and bioavailability compared with their nonprenylated parent molecules[10].

The synthesis of prenylflavonoids by chemical methods has always had many limitations, such as regio-/stereoselectivity and variations in the number of prenyl groups attached to various flavonoid skeletons. In contrast, enzymatic catalysis is effective and superior in terms of regioselectivity and stereoselectivity in the directional synthesis of prenylflavonoids. However, only a few flavonoid PTases from plants have been identified to date, and most of them are strictly substrate-specific and regiospecific[11–17]. It has been demonstrated that most of the enzymes involved in flavonoid scaffold biosynthesis, including chalcone synthase (CHS), chalcone isomerase (CHI), flavone synthase (FNS), isoflavone synthase (IFS), and flavanone 3β-hydroxylase (F3H) have broad substrate specificity[18,19]. Of these enzymes, CHI catalyzes the stereospecific isomerization of a chalcone into the corresponding (–)-flavanone *via* intramolecular cyclization with clear substrate flexibility[20,21]. To develop an effective method to synthesize prenylflavonoids, we focused on cascade reactions by combining chalcone PTase and CHI to produce prenylflavonoids with diverse structures (Scheme 1).

Licorice from the roots and rhizomes of various species of *Glycyrrhiza* (Leguminosae) is one of the most frequently used traditional medicines in China and in other countries[22,23]. More than 200 prenylated flavonoids have been identified in licorice, including prenylchalcones, prenylflavanones, prenylflavones, and prenylisoflavonoids[24,25]. Therefore, *Glycyrrhiza* is considered an ideal source for the discovery of biosynthetic enzymes/genes for the biosynthesis of prenylflavonoids. In this article, a new chalcone-specific PTase, GuILDT, and a new stereo- and regio-specific type II CHI, GuCHI, were characterized from *Glycyrrhiza uralensis* and employed for cascade reactions to produce diverse bioactive prenylflavonoids. Their enzymatic synthesis applications as well as roles they played in the biosynthesis of structurally different prenylflavonoids were also discussed.



Scheme 1 Biocatalytic access to diverse prenylflavonoids by combining various biosynthetic enzymes.

2. Materials and methods

2.1. General material and reagents

Plasmid pESC was purchased from Stratagene. Synthetic dextrose dropout medium lacking histone (SD-His) were used to select *Sacharomyces cerevisiae* transformants containing the assembled plasmids. Complex medium (YPDA) consists of 2% peptone, 1% yeast extract, and 2% glucose supplemented with 0.01% adenine hemisulfate. *S. cerevisiae* YPH499 was used as the host for DNA assembly and heterologous expression. Plasmid pET-28a was purchased from Invitrogen. The prenyl donors dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), and phytyl diphosphate (PPP) were chemically synthesized as describe previously[26]. Isoliquiritigenin (**1**), 2′-hydroxychalcone (**5**), and 2-hydroxychalcone (**6**) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Narigenin chalcone (4,2′,4′,6′-tetrahydroxychalcone, **2**), 2′,4′-dihydroxychalcone (**3**), 2,4,2′,4′-tetrahydroxychalcone (**4**), were chemically synthesized according to the literatures[27].

2.2. Plant material and culture conditions

G. uralensis callus cultures were maintained on Murashige and Skoog basal medium containing 30 g/L sucrose, 6 g/L agar, 0.2 mg/L α-naphthaleneacetic acid (NAA), 0.5 mg/L 6-benzylaminopurine (6-BA), and 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), pH 6.2[28]. The suspension of cultured cells were initiated by inoculating two-week-old callus cultures into 150 mL of Murashige and Skoog liquid medium, incubated on a rotary shaker at 120 rpm in the darkness at 25 °C, and subcultured every 15 days.

2.3. Analytical methods

^{1}H and ^{13}C NMR spectra were recorded on VNOVA SYSTEM-600 spectrometers using CD_3COCD_3 or CD_3OD as solvent. Optical rotations were determined using a Perkin-Elmer Model 341 LC polarimeter (Perkin-Elmer). Enzymatic products were analyzed with a 1200 HPLC system (Agilent) coupled with an LCQ Fleet ion trap mass spectrometer (Thermo Scientific). HPLC analyses were conducted with an RP-18 end-capped Purospher STAR LP column (250 mm × 4.6 mm, 5 µm, Merck Millipore) with a flow rate of 1 mL/min. UV detection was set at 290 or 380 nm, and the column was operated at 30 °C. The solvent system consisted of a linear gradient (45%–95%, *v*/*v*) of methanol in water

with formic acid (0.1%) over 20 min, followed by isocratic elution with methanol (100%) for 10 min. For LC–MS analyses, the LC effluent was introduced into an ESI source (post column splitting ratio, 2:1). Ultra-high purity helium was used as the collision gas, and high purity nitrogen was used as the nebulizing gas. The optimized parameters in the positive- and negative-ion modes were the following: sheath gas flow rate, 20 arbitrary units; auxiliary gas flow rate, 5 arbitrary units; spray voltage, 5.0 kV; capillary temperature, 350 °C; source collision induced decomposition (CID), 35 V; tube lens offset voltage, −75 V. For full-scan MS analysis, the spectra were recorded in the range *m/z* 100–900. For the isolation of enzymatic products, semi-preparative RP-HPLC was performed with a YMC-Pack ODS-A column (250 mm × 10 mm, 5 μm, YMC, Kyoto, Japan) at a flow rate of 3 mL/min with a linear gradient (40–80%, *v/v*) of acetonitrile in water over 15 min. The chiral analysis of flavanone enantiomers were operated utilizing a Chiralpak AD-H column (250 mm × 4.6 mm, 5 μm, Daicel Chemical Industries, Toyoko, Japan). The mobile phase consisted of *n*-hexane and 2-propanol (85:15, *v/v*), and analysed at 30 °C at a flow rate of 1.0 mL/min with detection at 290 nm.

2.4. cDNAs cloning of GuILDT

Total RNA was prepared from ten-day-old cultured cells of *G. uralensis* treated with methyl jasmonate (MJ, final concentration 0.1 mmol/L) using an E.Z.N.A. Plant RNA Kit (Omega Bio-Tek, Norcross, GA). First-strand cDNA was synthesized from 10 μg of total RNA in 20 μL with SmartScribe reverse transcriptase (Clontech, Mountain View, CA, USA).

For GuILDT isolation, a prime pair of 5′-AAATAGACAAGATAAAC-3′ and 5′-GTCAGGTATATCCTTGAAC-3′ was designed based on the sequence of GuA6DT and used to get about 400 bp nucleotides[16]. RT-products were subjected to rapid amplification of cDNA ends (RACE) according to the manufacture's protocol with 5′ and 3′ special primers: 5′-TGGCTTGGCTGGATTATAGGTTCATGGC-3′ and 5′-CAACAAGGGCACATTGATTGAATAGGC-3′. Finally, using the 5′ and 3′ sequences as specific primers (Forward primer: 5′-GAATTC ATGGATTCAATGGTTATTGGGTCTTTTCC-3′, the *EcoR I* site is underlined; Reverse primer: 5′-GCGGCCGCTCATCGAACTAAAGGTATGAGAAA′, the *Not I* site is underlined), the full-length cDNA GuILDT was amplified with Pfu DNA polymerase (TransGen Biotech). The PCR product was cloned into the pEASY-Blunt Simple Vector (TransGen Biotech) to create pEASY-*GuILDT*.

For isolation GuCHI, degenerate oligonucleotide primers were designed from highly conserved amino acid regions of known CHI sequences and named CHI-F1 (5′-GNACNTTYATHAARTTYAC-3′) and CHI-R1 (5′-GCRTGYTCNCCDATCAT-3′). To obtain the full-length sequences of CHI clone, the resulting cDNA was subjected to 5′- and 3′-RACE with (5′-AAAGGTGATTGAGAACAGTGTGGCC-3′ and 5′-TCCCATCTTGAGAGAAAACTAAGCCC-3′). Finally, using the 5′ and 3′ sequences as specific primers (Forward primer: 5′-GTTACATATGGCGGGAGCAGCACCAGTA-3′, the *Nde I* site is underlined; Reverse primer: 5′-AATCTGTCGACTCAGTTTCCGTTTCCAAT-3′, the *Sal I* site is underlined), the full-length cDNA GuCHI were amplified with Pfu DNA polymerase. The PCR product was cloned into the pEASY-Blunt Simple Vector to create pEASY-*GuCHI* and sequenced.

2.5. Heterologous expression of GuILDT in Sacharomyces cerevisiae

The yeast expression vector pESC and pEASY-*GuILDT* were digested by *EcoR* I and *Not* I, and then GuILDT and digested vector were ligated overnight at 16 °C with T4 ligase (NEB). The resulting plasmid, pESC-*GuILDT* was transformed into *S. cerevisiae* strain YPH499 by the modified LiCl method[29].

For the expression of recombinant protein, the transformant YPH499-pESC-*GuILDT* were cultured overnight at 30 °C in selective liquid media containing glucose as the carbon source. Cultures were collected by centrifugation, washed in sterile water, and re-suspended to an OD_{600} = 1.0 in fresh SG-His media containing galactose. After 24 h, the cells were harvested, re-suspended in extraction buffer (Tris–HCl 100 mmol/L, pH 9.0 containing phenylmethylsulfonylfluoride (PMSF, 1.0 mmol/L)) and homogenized with acid-washed glass beads eight times in a vortex mixer (each cycles: 30 s vortexing and 30 s cooling on ice). The homogenized mixture was centrifuged (10,000× *g*, 20 min), then the supernatant was ultracentrifuged (160,000× *g*, 90 min, 4 °C) to pellet the microsomal fraction. After washing, the membrane fraction was re-suspended with 100 mmol/L Tris–HCl buffer (pH 9.0), which was used as an enzyme solution for prenyltransferase assay described below. The total protein concentration was determined with the Bradford method using BSA as a standard[30].

2.6. Functional characterization of GuILDT in vitro

The basic enzyme assay was carried out with isoliquiritigenin (**1**, 200 μmol/L), DMAPP (400 μmol/L), and $MgCl_2$ (10 mmol/L). After incubation with yeast microsomes (200 μg of total protein) in a volume of 200 μL at 30 °C, the enzyme reaction was extracted with ethyl acetate (400 μL). The protein was removed by centrifugation (12,000× *g*, 15 min), the organic phase was evaporated to dryness under reduced pressure, and the residue was dissolved in methanol (200 μL) and used for HPLC–UV/ESI-MS analysis. To investigate the optimal pH, enzyme reaction was performed in sodium phosphate (pH 5.0−8.0) or Tris–HCl (pH 8.0−11.0) at 30 °C; To assay the optimal reaction temperature, the mixture was incubated at different temperatures (4−60 °C) in Tris–HCl buffer (100 mmol/L, pH 9.0); To test the requirement of GuILDT activity for divalent cations, 10 mmol/L $MgCl_2$, $BaCl_2$, $CaCl_2$, $FeCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, $MnCl_2$ and $NiCl_2$ were individually used with DMAPP and **1** in Tris–HCl buffer (100 mmol/L, pH9.0) at 30 °C for 1 h.

The apparent Michaelis–Menton constant (K_m) value for **1** was determined with yeast microsomes containing 80 μg of recombinant protein with varying concentrations (5, 10, 20, 40, 80, 160, and 400 μmol/L) and a fixed concentration of DMAPP (400 μmol/L), whereas the apparent K_m value for DMAPP was determined with varying concentrations of DMAPP (5, 10, 20, 40, 80, 160, and 400 μmol/L) and a fixed concentration of **1** (400 μmol/L). The reaction product was quantified using a standard curve generated from known concentrations of isobavachalcone (**1a**). Apparent *K*m values were calculated from Linewearver-Burk plots using Hyper32 software (http://homepage.ntlword.com/john.easterby/hyper32.html).

The prenyl acceptor specificity was studies in reaction mixtures containing microsome, various chalcones (200 μmol/L), including isoliquiritigenin (2,2′,4′-trihydroxychalcone, **1**), naringenin chalcone (4,2′,4′,6′-tetrahydroxychalcone, **2**), 2′,4′-dihydroxychalcone (**3**),

2,4,2′,4′-tetrahydroxychalcone (**4**), 2-hydroxychalcone (**5**), or 2′-hydroxychalcone (**6**), with DMAPP as prenyl donor (400 μmol/L). The prenyl donor specificity was tested with DMAPP, GPP, FPP, GGPP, or PPP (1 mmol/L), with isoliquiritigenin (**1**) as prenyl acceptor (200 μmol/L).

Four chalcones (**1**–**4**) were used as prenyl acceptors and incubated with DMAPP and recombinant enzyme for products preparation, respectively. The enzymatic reaction mixture contained $MgCl_2$ (10 mmol/L), prenyl acceptor (2 mmol/L), DMAPP (4 mmol/L), and yeast microsomal protein (10–20 mg) in a total volume of 10–15 mL. The enzyme products **1a**, **3a**, and **4a** (0.2–1.2 mg) were obtained by semi-preparative RP-HPLC and analyzed by MS, ^{1}H and ^{13}C NMR spectroscopy. **2a** was confirmed with the standard compound synthesized according the previous report[31,32].

2.7. *Heterologous expression in Escherichia coli and protein purification of GuCHI*

GuCHI was inserted into the pET-28a bacterial expression vector at the *Nde I* and *Sal I* sites. His_6-GuCHI was heterologously expressed in Rosetta (DE3) *E. coli* cells (Novagen, Madison, WI, USA). Bacterial cultures were grown in LB media containing 34 mg/L chloromycetin and 50 mg/L kanamycin overnight at 37 °C. The cultures were diluted and then grown to an OD_{600} 0.4–0.6. At this point, expression of the recombinant protein was induced by addition of 0.1 mmol/L isopropyl-thio-*β*-D-galactoside (IPTG). After incubated overnight at 16 °C, cultures were harvested by centrifugation at 4000× *g* for 10 min and washed with sterile water. After washing, collected cultures was frozen with liquid nitrogen and stored at −80 °C.

The cell pellet was suspended in 30 mL lysis buffer (20 mmol/L sodium-phosphate, pH 8.0, 500 mmol/L NaCl, 1 mmol/L PMSF), and lysed through sonication on ice. Followed by sonication and centrifugation, the cleared cell lysate was directly applied on to a column that was packed with Ni-NTA agarose. After washed with the buffer (20 mmol/L sodium-phosphate, pH 7.5, 500 mmol/L NaCl, 50 mmol/L imidazole), the protein was eluted with the buffer (20 mmol/L sodium-phosphate, pH 7.5, 500 mmol/L NaCl, 150 mmol/L imidazole). Purified protein His_6-GuCHI was concentrated and the buffer exchanged into reaction buffer (50 mmol/L Tris–HCl, 50 mmol/L NaCl, 1 mmol/L DTT, 1% glycerol, pH 7.5). The concentrated enzyme solutions were aliquot, flash frozen, and stored at −80 °C.

2.8. *Functional assay of GuCHI in vitro*

Chalcones (**1**–**4**) and their prenylated derivatives (**1a**–**4a**) (40 mmol/L) were incubated with 200 μL of reaction buffer at 25 °C for 5 min, extracted with ethyl acetate, and analyzed by HPLC, respectively. For prepared the cyclized products, chalcones and their prenylated derivatives were added into the reaction buffer containing purified His_6-GuCHI protein. After incubation at 25 °C for 15 min, the reaction mixtures were extracted with ethyl acetate. The extracts were subjected to semi-preparative reversed HPLC chromatography, respectively.

For kinetic properties study, the decrease of substrate A395 was monitor using a nano detector. Spectrophotometric operations were performed at 25 °C in 0.5 mL reaction buffer. Reactions were initiated by the addition of enzyme, and initial rates were calculated based upon decrease of substrate A395. Steady-state kinetic parameters (K_m) were calculated by fitting the untransformed initial velocity data.

2.9. *Chemical and physical data of products **1a–4a, 1b–4b**, and **1c–4c***

Isobavachalcone (**1a**)[33]: ESI-MS: *m/z* 325 $[M + H]^+$; ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 1.64 (3H, s, H-5″), 1.78 (3H, s, H-4″), 3.35 (2H, d, J = 7.2 Hz, H-1″), 5.27 (1H, t, J = 7.2 Hz, H-2″), 6.52 (1H, d, J = 9.0 Hz, H-5′), 6.92 (2H, d, J = 8.4 Hz, H-3 and H-5), 7.73 (2H, d, J = 8.4 Hz, H-2 and H-6), 7.75 (1H, d, J = 15.6 Hz, H-*α*), 7.83 (1H, d, J = 15.6 Hz, H-β), 7.97 (1H, d, J = 9.0 Hz, H-6′), 9.10 (4-OH), 9.44 (4′-OH), 13.99 (2′-OH); ^{13}C NMR (CD_3COCD_3, 150 MHz) δ_C 17.8 (C-4″), 22.0 (C-1″), 25.9 (C-5″), 95.2 (C-5′), 105.6 (C-1′), 108.0 (C-3′), 116.7 (C-3), 116.7 (C-5), 124.2 (C-2″), 125.5 (C-*α*), 128.2 (C-1), 130.7 (C-3″), 131.1 (C-2), 131.1 (C-6), 143.0 (C-β), 160.2 (C-2′), 160.5 (C-6′), 162.8 (C-4′), 165.9 (C-4), 193.4 (C=O).

Desmethylxanthol (**2a**)[31,32]: ESI-MS: *m/z* 341 $[M + H]^+$; ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 1.62 (3H, s, H-5″), 1.74 (3H, s, H-4″), 3.25 (2H, d, J = 6.6 Hz, H-1″), 5.23 (1H, t, J = 6.6 Hz, H-2″), 6.11 (1H, s, H-5′), 6.89 (2H, d, J = 9.0 Hz, H-3 and H-5), 7.55 (2H, d, J = 9.0 Hz H-2 and H-6), 7.75 (1H, d, J = 15.6 Hz, H-*α*), 7.89 (1H, d, J = 9.0 Hz, H-6′), 8.12 (1H, d, J = 15.6 Hz, H-β); ^{13}C NMR (CD_3COCD_3, 150 MHz) δ_C 17.8 (C-4″), 22.0
(C-1″), 25.9 (C-5″), 95.2 (C-5′), 105.6 (C-1′), 108.0 (C-3′), 116.7 (C-3), 116.7 (C-5), 124.2 (C-2″), 125.5 (C-*α*), 128.1 (C-1), 130.7 (C-3″), 131.1 (C-2), 131.1 (C-6), 143.0 (C-β), 160.2 (C-2′), 160.5 (C-6′), 162.8 (C-4′), 165.9 (C-4), 193.4 (C=O).

3′-Dimethylallyl-2′,4′-dihydroxychalcone (**3a**)[34]: ESI-MS: *m/z* 309 $[M + H]^+$; ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 1.65 (3H, s, H-5″), 1.78 (3H, s, H-4″), 3.38 (2H, d, J = 7.2 Hz, H-1″), 5.28 (1H, t, J = 7.2 Hz, H-2″), 6.55 (1H, dd, J = 8.4, 2.4 Hz, H-5′), 7.46 (3H, m, overlapped, H-2, H-4 and H-6), 7.85 (2H, m, overlapped, H-3 and H-5), 7.87 (1H, dd, J = 15.6, 2.4 Hz, H-*α*), 7.95 (1H, d, J = 15.6 Hz, H-β), 8.02 (1H, d, J = 8.4 Hz, H-6′), 13.84 (2′-OH); ^{13}C NMR (CD_3COCD_3, 150 MHz) δ_C 18.0 (C-4″), 22.4 (C-1″), 26.0 (C-5″), 108.3 (C-5′), 114.5 (C-1′), 116.3 (C-3′), 122.0 (C-*α*), 123.3 (C-2″), 129.7 (C-2), 129.7 (C-6), 129.9 (C-3), 129.9 (C-5), 130.7 (C-6′), 131.5 (C-4), 131.7 (C-1), 136.1 (C-3″), 144.6 (C-β), 163.2 (C-4′), 165.4 (C-2′), 193.4 (C=O).

Morachalcone A (**4a**)[35]: ESI-MS: *m/z* 341 $[M + H]^+$; ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 1.64 (3H, s, H-5″), 1.78 (3H, s, H-4″), 3.37 (2H, d, J = 7.2 Hz, H-1″), 5.28 (1H, t, J = 7.2 Hz, H-2″), 6.46 (1H, dd, J = 8.4, 2.4 Hz, H-5), 6.51 (1H, d, J = 2.4 H-3), 6.52 (1H, d, J = 9.0 Hz, H-5′), 7.69 (1H, d, J = 8.4 Hz, H-6), 7.79 (1H, d, J = 15.6 Hz, H-*α*), 7.89 (1H, d, J = 9.0 Hz, H-6′), 8.21 (1H, dd, J = 15.6, 2.4 Hz, H-β), 8.88 (OH), 9.19 (OH), 9.24 (OH), 14.14 (2′-OH); ^{13}C NMR (CD_3COCD_3, 150 MHz) δ_C 18.0 (C-4″), 22.4 (C-1″), 25.9 (C-5″), 103.7 (C-3), 107.9 (C-5′), 109.3 (C-5), 114.6 (C-1′), 114.9 (C-1), 115.4 (C-3′), 117.7 (C-β), 123.5 (C-2″), 130.0 (C-6′), 131.5 (C-6), 131.8 (C-1″), 140.9 (C-*α*), 160.0 (C-2), 162.3 (C-4), 165.2 (C-4′), 193.6 (C=O).

(–)-Liquiritigenin (**1b**)[33]: $[\alpha]_D^{20}$: –66.7 (*c* 0.15, MeOH); ^{1}H NMR (CD_3COCD_3, 400 MHz) δ_H 2.69 (1H, dd, J = 16.8, 2.4 Hz, H-3a), 3.05 (1H, dd, J = 16.8, 13.2 Hz, H-3b), 5.39 (1H, dd, J = 13.2, 2.4 Hz, H-2), 6.36 (1H, d, J = 2.4 Hz, H-8), 6.50 (1H, dd, J = 8.8, 2.4 Hz, H-6), 6.82 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 7.33 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 7.73 (1H, d, J = 8.8 Hz, H-5); ^{13}C NMR (CD_3OD, 100 MHz) δ_C 45.1 (C-3), 81.2 (C-2),

104.0 (C-10), 111.9 (C-8), 115.1(C-4a), 116.5 (C-3′ and C-5′), 129.2 (C-2′ and C-6′), 130.0 (C-6), 131.5 (C-5), 159.1 (C-4′), 165.7 (C-8a), 167.0 (C-7), 193.7 (C-4).

(–)-Naringenin ((–)-**2b**)[36]: $[\alpha]_D^{20}$: –26.7 (*c* 0.15, MeOH); ^{1}H NMR (CD_3COCD_3, 400 MHz) δ_H 2.74 (1H, dd, J = 17.2, 3.2 Hz, H-3a), 3.18 (1H, dd, J = 17.2, 13.2 Hz, H-3b), 5.46 (1H, dd, J = 13.2, 2.8 Hz, H-2), 5.95 (1H, d, J = 2.0 Hz, H-6), 5.96 (1H, d, J = 2.0 Hz, H-8), 6.90 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 7.39 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 12.18 (5-OH); ^{13}C NMR (CD_3COCD_3, 100 MHz) δ_C 44.1 (C-3), 80.5 (C-2), 96.4 (C-8), 97.4 (C-6), 103.8 (C-4a), 116.7 (C-3′), 116.7 (C-5′), 129.6 (C-2′), 129.6 (C-6′), 131.4 (C-1′), 159.3 (C-4′), 164.9 (C-8a), 165.9 (C-5), 167.9 (C-7), 197.8 (C-4).

(–)-7-Hydroxyflavanone (**3b**)[37]: $[\alpha]_D^{20}$: –82.1 (*c* 0.39, MeOH); ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 2.75 (1H, dd, J = 16.2, 3.0 Hz, H-3a), 3.04 (1H, dd, J = 16.2, 12.6 Hz, H-3b), 5.57 (1H, dd, J = 13.2, 2.8 Hz, H-2), 6.46 (1H, d, J = 1.8 Hz, H-8), 6.59 (1H, dd, J =9.0, 1.8 Hz, H-6), 7.39 (1H, m, H-4′), 7.45 (2H, t, J = 7.2 Hz, H-3′ and H-5′), 7.58 (2H, d, J = 7.2 Hz, H-2′ and H-6′), 7.74 (1H, d, J = 9.0 Hz, H-5), 9.43 (1H, s, 7-OH); ^{13}C NMR (CD_3COCD_3, 150 MHz) δ_C 44.9 (C-3), 80.7 (C-2), 103.8 (C-8), 111.4 (C-6), 115.4 (C-4a), 127.4 (C-2′ and C-6′), 129.4 (C-4′), 129.5 (C-3′ and C-5′), 129.6 (C-5), 140.6 (C-1′), 164.4 (C-8a), 165.4 (C-7), 190.2 (C-4).

(–)-7,2′,4′-Trihydroxyflavanone (**4b**)[38]: $[\alpha]_D^{20}$: –18.8 (*c* 0.16, MeOH); ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 2.68 (1H, dd, J = 16.8, 3.0 Hz, H-3a), 3.03 (1H, dd, J = 16.8, 13.2 Hz, H-3b), 5.72 (1H, dd, J = 13.2, 3.0 Hz, H-2), 6.43 (1H, d, J = 2.4 Hz, H-3′), 6.44 (1H, dd, J = 8.4, 2.4 Hz, H-4′), 6.47 (1H, d, J = 2.4 Hz, H-8), 6.57 (1H, dd, J = 8.4, 2.4 Hz, H-6), 7.34 (1H, d, J = 8.4 Hz, H-6′), 7.74 (1H, d, J = 8.4 Hz, H-5). ^{13}C NMR (CD_3COCD_3, 150 MHz) δ_C 43.9 (C-3), 75.9 (C-2), 103.5 (C-8), 103.7 (C-3′), 107.9 (C-6), 111.2 (C-1′), 115.3 (C-4a), 118.0 (C-5′), 129.0 (C-6′), 129.6 (C-5), 156.3 (C-2′), 159.5 (C-4′), 165.0 (C-8a), 165.3 (C-7), 191.1 (C-4).

(–)8-Dimethylallylliquiritigenin (**1c**)[39]: $[\alpha]_D^{20}$: –57.3 (*c* 0.18, MeOH); ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 1.62 (3H, s, H-5″), 1.63 (3H, s, H-4″), 2.69 (1H, dd, J = 16.8, 2.4 Hz, H-3a), 3.00 (1H, dd, J = 16.8, 13.2 Hz, H-3b), 3.33 (2H, brd, J = 7.2 Hz, H-1″), 5.23 (1H, t, J = 7.2 Hz, H-2″), 5.45 (1H, dd, J = 13.2, 2.4 Hz, H-2), 6.62 (1H, d, J = 8.4 Hz, H-6), 6.90 (2H, d, J = 7.8 Hz, H-3′ and H-5′), 7.42 (2H, d, J = 7.8 Hz, H-2′ and H-6′), 7.59 (1H, d, J = 8.4 Hz, H-5); ^{13}C NMR (CD_3COCD_3, 150 MHz) δ_C 18.0 (C-4″), 22.9 (C-1″), 26.0 (C-5″), 44.6 (C-3), 80.4 (C-2), 110.5 (C-6), 115.5 (C-8), 116.2 (C-3′ and C-5′), 116.6 (C-4a), 123.2 (C-2″), 126.4 (C-5), 128.9 (C-2′ and C-6′), 131.6 (C-1′), 131.7 (C-3″), 158.5 (C-4′), 162.0 (C-8a),162.3 (C-7),191.0 (C-4).

(–)-8-Dimethylallylnaringenin ((–)-**2c-A**)[40]: $[\alpha]_D^{20}$: –20.1 (*c* 0.15, MeOH); ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 1.59 (3H, s, H-5″), 1.60 (3H, s, H-4″), 2.75 (1H, dd, J = 16.8, 2.7 Hz, H-3a), 3.13 (1H, dd, J = 16.8, 12.6 Hz, H-3b), 3.21 (2H, brd, J = 7.2 Hz, H-1″), 5.19 (1H, t, J = 7.2 Hz, H-2″), 5.45 (1H, dd, J = 12.6, 2.7 Hz, H-2), 6.04 (1H, s, H-6), 6.90 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 7.41 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 8.50 (4′-OH), 9.57 (7-OH), 12.14 (5-OH); ^{13}C NMR (CD_3COCD_3, 150 MHz) δ_C 17.9 (C-4″), 22.3 (C-1″), 26.0 (C-5″), 43.5 (C-3), 79.8 (C-2), 94.5 (C-6), 103.3 (C-4a), 108.4 (C-8), 116.2 (C-3′ and C-5′), 123.8 (C-2″), 128.9 (C-2′ and C-6′), 131.2 (C-1′), 131.3 (C-2″), 158.7 (C-4′), 161.2 (C-5), 162.8 (C-8a), 163.1 (C-7),197.6 (C-4).

6-Dimethylallylnaringenin ((–)-**2c-B**)[41]: ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 1.63 (3H, s, H-5″), 1.74 (3H, s, H-4″), 2.72 (1H, dd, J = 16.8, 2.7 Hz, H-3a), 3.18 (1H, dd, J = 16.8, 12.6 Hz, H-3b), 3.24 (2H, brd, J = 7.2 Hz, H-1″), 5.21 (1H, t, J = 7.2 Hz, H-2″), 5.42 (1H, dd, J = 12.6, 3.0 Hz,H-2), 6.02 (1H, s, H-6), 6.89 (2H, d, J = 6.4 Hz, H-3′ and H-5′), 7.38 (2H, d, J = 6.4 Hz, H-2′ and H-6′), 8.49 (4′-OH), 9.53 (7-OH), 12.46 (5-OH).

(–)-8-Dimethylallyl-7-hydroxyflavanone (**3c**)[42]: $[\alpha]_D^{20}$: –42.3 (*c* 0.14, MeOH); ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 1.62 (3H, s, H-5″), 1.63 (3H, s, H-4″), 2.77 (1H, dd, J = 16.8, 3.0 Hz, H-3a), 3.00 (1H, dd, J = 16.8, 12.6 Hz, H-3b), 3.37 (2H, t, J = 7.2 Hz, H-1″), 5.25 (1H, t, J = 7.2 Hz, H-2″), 5.57 (1H, dd, J = 12.6, 3.0 Hz, H-2), 6.64 (1H, d, J = 8.4 Hz, H-6), 7.39 (1H, brt, J = 7.2 Hz, H-4′), 7.46 (2H, brt, J = 7.2 Hz, H-3′ and H-5′), 7.60 (2H, overlapped, H-2′ and H-6′), 7.61 (1H, overlapped, H-5), 9.28 (7-OH). ^{13}C NMR (CD_3COCD_3, 600 MHz) δ_C 18.0 (C-4″), 22.9 (C-1″), 26.0 (C-5″), 44.8 (C-3), 80.5 (C-2), 110.6 (C-6), 115.5 (C-4a), 116.6 (C-8), 123.2 (C-2″), 126.4 (C-5), 127.2 (C-3′ and C-5′), 129.2 (C-4′), 129.5 (C-2′ and C-6′), 131.8 (C-3″), 140.9 (C-1′), 161.9 (C-8a), 162.4 (C-7), 190.6 (C-4).

Euchrenone a7 (**4c**)[43]: ^{1}H NMR (CD_3COCD_3, 600 MHz) δ_H 1.63 (3H, s, H-5″), 1.67 (3H, s, H-4″), 2.72 (1H, dd, J = 16.8, 3.0 Hz, H-3a), 2.98 (1H, dd, J = 16.8, 13.2 Hz, H-3b), 3.37 (2H, t, J = 7.2 Hz, H-1″), 5.27 (1H, t, J = 7.2 Hz, H-2″), 5.71 (1H, dd, J = 13.2, 3.0 Hz, H-2), 6.45 (1H, dd, J = 8.4, 2.4 Hz, H-5′), 6.49 (1H, d, J = 2.4 Hz, H-3′), 6.64 (1H, d, J = 8.4 Hz, H-6), 7.38 (1H, brt, J = 8.4 Hz, H-6′), 7.61 (1H, d, J = 8.4 Hz, H-5), 9.35 (7-OH), 8.68 and 8.42 (4′-OH and 2′-OH).

3. Results and discussion

In an effort to find the chalcone-specific PTase(s) gene in *Glycyrrhiza*, homology-based RT-PCR/RACE was performed[12,16,17]. One candidate PTases cDNA was isolated from the cultured cells of *G. uralensis* and encoded a polypeptide of 408 amino acids. The polypeptide was clustered into plant flavonoid PTases phylogenic clade and shared 53% identity with the previously reported plant flavonoid PTases (Fig. 1). Two conserved aspartate-rich motifs of plant PTases including NQXXDXXXD and KD(I/L)XDX(E/D)GD were observed, as well as nine putative transmembrane regions (Supporting information Fig. S1).

To investigate its prenylation activity with chalcone, the putative protein was heterologously expressed in the yeast YPH499. A microsomal fraction was prepared and incubated with isoliquiritigenin (**1**, molecular weight (MW) 256) and dimethylallyl diphosphate (DMAPP) in the presence of $MgCl_2$. The enzymatic reaction (100 mmol/L Tris–HCl, pH 9.0; 0.2 mmol/L **1**; 0.4 mmol/L DMAPP; 400 μg of microsomes containing recombinant protein; 30 °C, 12 h) was analyzed by HPLC–ESI/MS, which gave an ion peak $[M - H]^-$ at *m/z* 323, with 68 amu larger MW than that of **1**, indicating the formation of a mono-prenylated product (Fig. 2). The product (**1a**) was further prepared in a scaled-up reaction and identified by NMR. A comparison of the ^{1}H NMR spectra of **1a** and **1** revealed that one dimethylallyl moiety had been introduced and that one methylene proton signal at δ_H 3.35 (d, J = 7.2 Hz, H_2-1″), one olefinic proton signal at δ_H 5.27 (t, J = 7.2 Hz, H-2″), and two methyl signals at δ_H 1.78 and 1.64 (H_3-4″ and H_3-5″) had appeared. The disappearance of H-3′ of **1** suggested the dimethylallyl moiety was attached at the *C*-3′ position. In addition, the downfield shift of *C*-3′ from δ_C 99.8 in **1** to δ_C 108.0 in **1a** further confirmed that deduction. Thus, the structure of **1a** was unambiguously determined as isobavachalcone[44], and the enzyme was designated GuILDT (*G. uralensis* isoliquiritigenin dimethylallyltransferase, GenBank accession code KR139751).

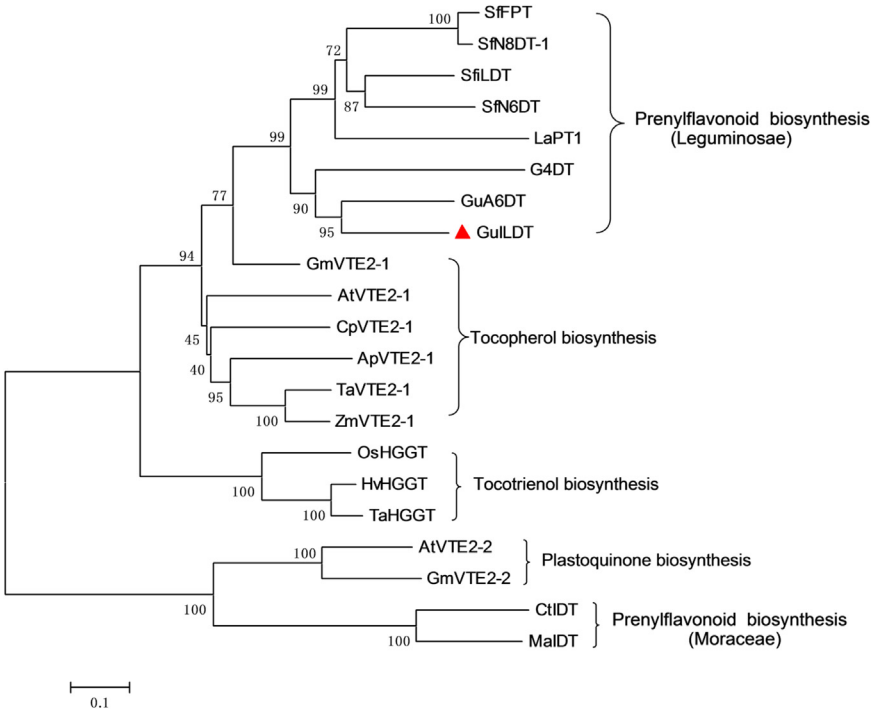


Figure 1 The phylogenetic relationships between the putative GuILDT protein and related plant prenyltransferases. The protein sequences were aligned using ClustalW. The neighbor-joining phylogenetic tree was drawn using MEGA 5.0. The bootstrap values are 1000 and the branch lengths represent the relative genetic distances. The abbreviation of the protein sequences and their accession numbers are as follows: GuILDT (*Glycyrrhiza uralensis*; KR139751); GuA6DT (*G. uralensis*; KJ123716); SfFPT (*Sophora flavescens*; KC513505); SfN8DT-1 (*S. flavescens*; AB325579); SfG6DT (*S. flavescens*; BAK52291); SfiLDT (*S. flavescens*; AB604223); LaPT1 (*Lupinus albus*; JN228254); G4DT (*Glycine max*; AB434690); GmVTE2-1 (*G. max*; DQ231059); AtVTE2-1 (*Arabidopsis thaliana*; AY089963); CpVTE2-1 (*Cuphea pulcherrima*; DQ231058); ApVTE2-1 (*Allium porrum*; DQ231057); TaVTE2-1 (*Triticum aestivum*; DQ231056); ZmVTE2-1 (*Zea mays*; DQ231055); OsHGGT (*Oryza sativa*; AY222862); HvHGGT (*Hordeum vulgare*; AY222860); TaHGGT (*T. aestivum*; DQ231056); AtVTE2-2 (*A. thaliana*; DQ231060); GmVTE2-2 (*G. max*; DQ231061); CtIDT (*C. tricuspidata*; KM262660); and MaIDT (*M. alba*; KM262659).

To determine the substrate selectivity of GuILDT, 6 chalcones, which included **1**, naringenin chalcone (**2**), 2′,4′-dihydroxychalcone (**3**), 2,4,2′,4′-tetrahydroxychalcone (**4**), 2-hydroxychalcone (**5**), and 2′-hydroxychalcone (**6**), and 13 other flavonoids of different types were tested as prenyl acceptors (Fig. 2 and Supporting information Fig. S2). The results of the enzymatic assay indicated that compounds **1**–**4** were recognized by GuILDT but the other flavonoids were not (Supporting information Fig. S3), which indicated that GuILDT is a chalcone-specific PTase and that the 4′-hydroxyl is critical for prenylation. Product **2a** was confirmed with the authentic compound desmethylxanthohumol due to its spontaneous cyclizing to (±)-**2c**-**A** and (±)-**2c**-**B** (Fig. 2). Compounds **3a** and **4a** were identified as the *C*-3′ mono-prenyl derivatives by MS and NMR spectroscopy (Fig. 2, See Supporting information for details), indicating that GuILDT was a *C*-3′ regio-specific chalcone PTase. In terms of the prenyl donor specificity, only DMAPP was accepted in the incubation. No detectable prenylated products were observed with geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), and phytyl pyrophosphate (PPP) (Supporting information Fig. S3). An investigation of the effects of divalent cations on the GuILDT activity revealed that this activity was divalent cation-dependent. The maximal activity was observed in the presence of Mg^{2+}, and the relative activities were calculated as 12%–96% when Co^{2+}, Ni^{2+}, Fe^{2+}, Ba^{2+}, Mn^{2+}, and Ca^{2+} were added (Supporting information Fig. S4A). The optimum reaction temperature was approximately 30 °C (Supporting information Fig. S4B), and the optimal pH value was 9.0 in 100 mmol/L Tris–HCl (Supporting information Fig. S4C). Furthermore, the *K*m value of **1** was 16 μmol/L, and the K_m of DMAPP was 138 μmol/L (Supporting information Fig. S5).

Furthermore, great effort was made to identify a prenylchalcone-recognized CHI from the cell cultures of *G. uralensis*. In nature, CHI catalyzes the stereo-specific isomerization of chalcones into the corresponding (−)-flavanones as intermediates of subsequent flavonoid biosynthesis[45–47]. However, this reaction can occur chemically and yields two enantiomers, (+)- and (−)-flavanones. A homology-based PCR/RACE strategy was used to obtain a full-length CHI cDNA from cultured *G. uralensis* cells based on two conserved amino acid sequences, GXFXKFT and (M/I)IGXH(G/A). This sequence contained an open reading frame (690 bp) encoding a polypeptide of 229 amino acids that showed a 43%–53% identity with various type I CHIs and 74%–80% with type II CHIs (Supporting information Fig. S6). It was clustered into the latter group and designated as GuCHI (*G. uralensis* chalcone isomerase, GenBank accession code KR139752, Fig. 3).

The recombinant GuCHI was heterologously expressed in *E. coli* as a His_6-tagged protein and subsequently purified by affinity chromatography for enzymatic assays. The incubation of **1** with GuCHI (50 mmol/L Tris–HCl, 50 mmol/L NaCl, 1 mmol/L DTT, 1% glycerol, pH 7.5, 25 °C, 5 min) yielded one product, which was identified as (−)-liquiritigenin (**1b**) by NMR and CD spectroscopy. Additionally, GuCHI can also catalyze the reaction of other chalcones (**2**–**4**) to the corresponding flavanones (Fig. 4, see Supporting information for details). It is noteworthy that **2** can be spontaneously isomerized into (±)-naringenin in aqueous solution. A chiral HPLC analysis of the

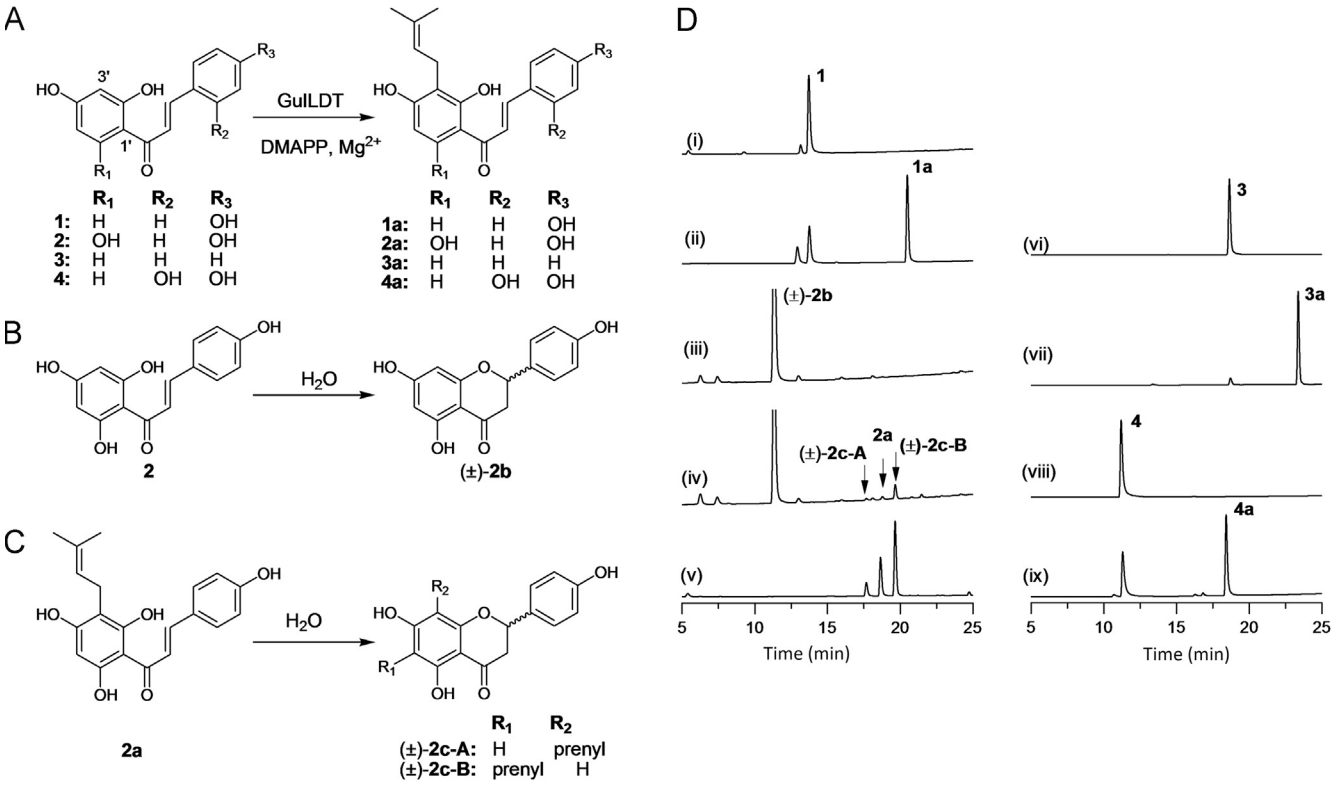


Figure 2 Prenylation functional analyses of recombinant GuILDT. (A) GuILDT prenylation; (B–C) Spontaneous cyclization of **2** and **2a** in basic solution, respectively; (D) HPLC chromatograms of reactions. Control assays lacking GuILDT with **1** (i), **2** (iii), **3** (vi), and **4** (viii). Compound **2** transformed to (±)-**2b** spontaneously in reaction buffer after a 12 h's incubation (iii). GuILDT assays with **1** (ii), **2** (iv), **3** (vii), and **4** (ix). Compound **2a** isomerized into (±)-**2c**-**A** and (±)-**2c**-**B** spontaneously in the reaction buffer (v). HPLC analyses of (i), (ii), and (vi)–(iix) were monitored at 380 nm, (iii)–(v) were monitored at 290 nm.

products gave (−)-naringenin in 90% yield, indicating the predominant contribution of enzymatic cyclization by GuCHI in this process (Supporting information Fig. S7A).

Based on the availability of a promiscuous GuCHI and prenylated chalcones **1a**–**4a**, the enzymatic isomerization of **1a**–**4a** were performed with GuCHI. Products **1c**–**4c** with prenyl substituents

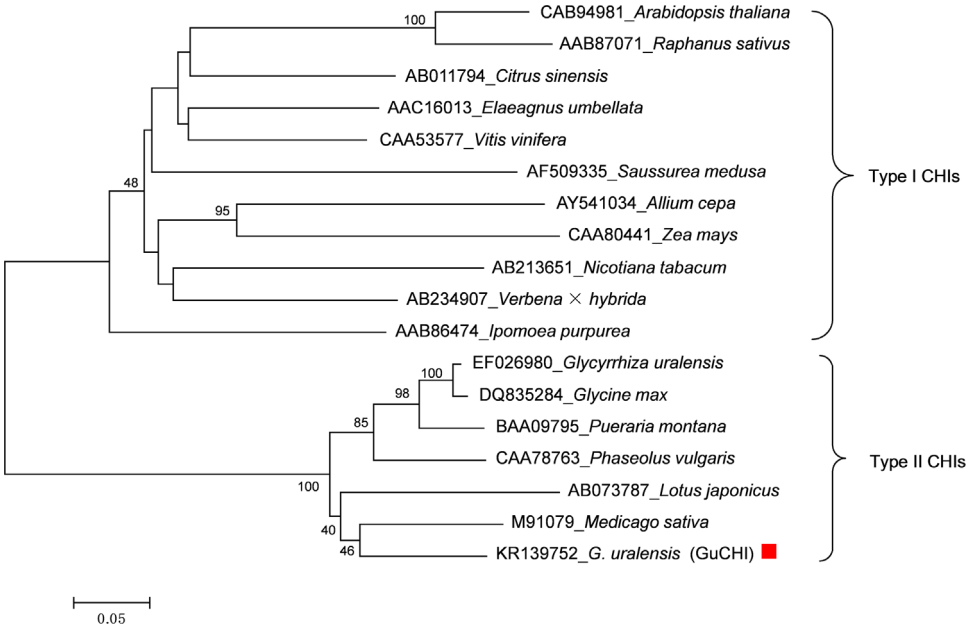


Figure 3 The phylogenetic relationships between the putative GuCHI protein and related CHIs. The sequences were aligned using ClustalW. The neighbor-joining phylogenetic tree was drawn using MEGA 5.0. The bootstrap values are 1000 and the branch lengths represent the relative genetic distances.

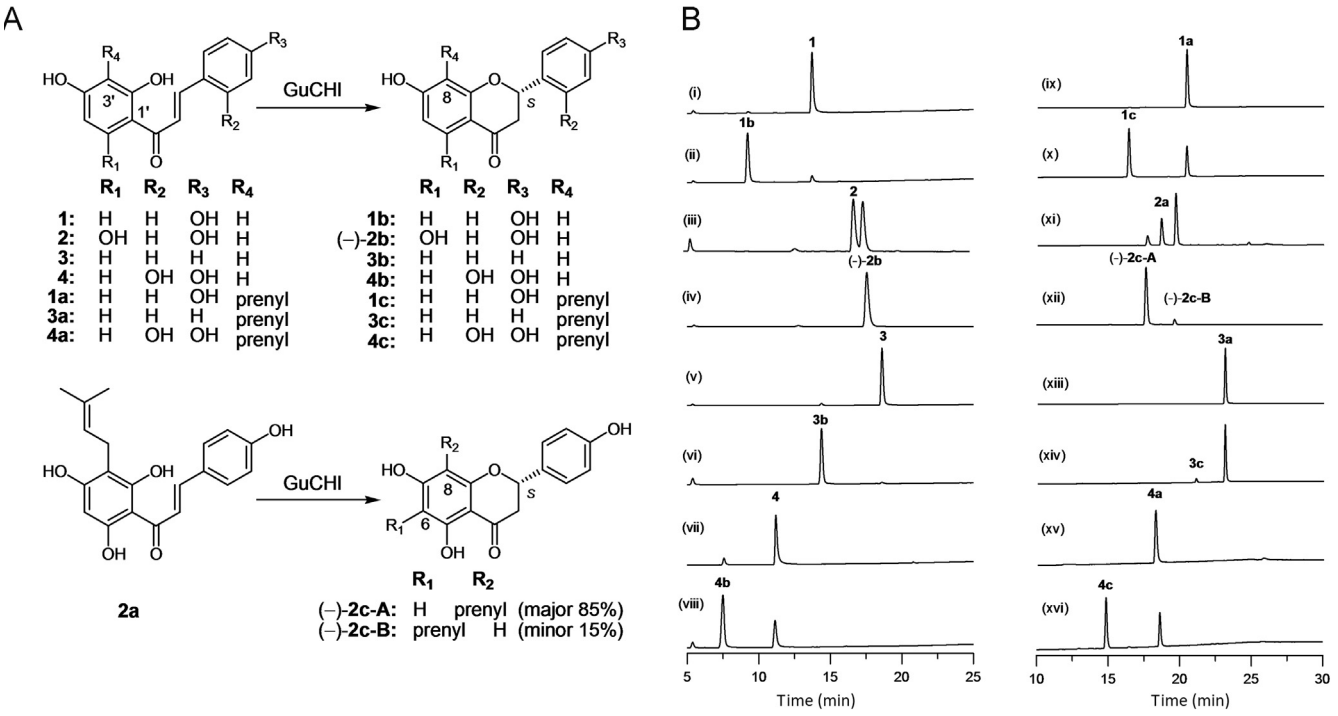


Figure 4 Cyclization functional analysis of GuCHI with chalcones **1**–**4** and their prenylated derivatives **1a**–**4a**. (A) GuCHI cyclization; (B) HPLC chromatograms of the reactions. Control assays lacking GuCHI with **1** (i), **2** (iii), **3** (v), **4** (vii), **1a** (ix), **2a** (xi), **3a** (xiii), and **4a** (xv); GuCHI assays with **1** (ii), **2** (iv), **3** (vi), **4** (viii), **1a** (x), **2a** (xii), **3a** (xiv), and **4a** (xvi). HPLC analyses were monitored at 290 nm.

were observed and prepared, and their structures were identified as the corresponding (−)-prenylflavanones by MS, ^{1}H NMR, and ^{13}C NMR spectroscopy (Fig. 4, see Supporting information for details). Both (−)-8-prenylnaringenin ((−)-**2c**-**A**, in 85% yield with 98% *ee* value) and (−)-6-prenylnaringenin((−)-**2c**-**B**, in 15% yield with 56% *ee* value) were generated when **2a** was reacted with GuCHI, which indicated that the isomerization by GuCHI is stereo- and regio-specific (Supporting information Fig. S7B–C). Notably, enzymatic catalysis played a vital role in the regio-specific cyclization of levorotatory flavanone, although spontaneous cyclization was also observed. GuCHI exhibited substrate flexibility and accepted a broad range of chalcones with either different hydroxyl substitution patterns in ring A or *C*-3′/*C*-5′ prenylation in ring A′. However, the cyclization of prenylated chalcone gave much slower kinetics than did a non-prenylated chalcone. For example, the K_m values of **1** and **2** were 15 and 3 μmol/L, respectively, and that of **1a** and **2a** were 32 and 11 μmol/L, respectively (Supporting information Fig. S8).

In summary, in *G. uralensis*, we characterized GuILDT, a new membrane-bound chalcone-specific PTase with *C*-3′ regio-specificity, and GuCHI, a new stereo- and regio-specific type II CHI with substrate promiscuity. GuCHI is the first reported CHI that catalyzes the intramolecular cyclization of prenylchalcones to generate prenylflavanones. The results reported herein provide a new approach to the enzymatic synthesis of prenylflavonoids and create the possibility of producing diverse bioactive natural/unnatural prenylflavonoids by enzymatic synthesis, even by combinatorial biocatalysis with other enzymes. In addition, considering the checkpoint role of chalcones (naringenin chalcone and isoliquiritigenin) in the flavonoid biosynthetic pathway and the promiscuity of the tailoring enzymes[45–47], the results suggest that the prenyl moiety might also been introduced at the chalcone stage to yield prenylchalcones, which subsequently act as common intermediates involved in the biosynthesis of a variety of structurally different prenylflavonoids.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2018.01.009.

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