Characterization of UDP-glucose dehydrogenase isoforms in the medicinal legume *Glycyrrhiza uralensis*

Ayumi Kawasaki, Ayaka Chikugo, Keita Tamura, Hikaru Seki, Toshiya Muranaka*

Department of Biotechnology, Graduate School of Engineering, Osaka University, Osaka 565-0871, Japan

*E-mail: muranaka@bio.eng.osaka-u.ac.jp  Tel: +81-6-6879-7423  Fax: +81-6-6879-7426

Received June 15, 2020; accepted February 22, 2021 (Edited by S. Takahashi)

Abstract

Uridine 5′-diphosphate (UDP)-glucose dehydrogenase (UGD) produces UDP-glucuronic acid from UDP-glucose as a precursor of plant cell wall polysaccharides. UDP-glucuronic acid is also a sugar donor for the glycosylation of various plant specialized metabolites. Nevertheless, the roles of UGDs in plant specialized metabolism remain poorly understood. *Glycyrrhiza* species (licorice), which are medicinal legumes, biosynthesize triterpenoid saponins, soyasaponins and glycyrrhizin, commonly glucuronosylated at the C-3 position of the triterpenoid scaffold. Often, several different UGD isoforms are present in plants. To gain insight into potential functional differences among UGD isoforms in triterpenoid saponin biosynthesis in relation to cell wall component biosynthesis, we identified and characterized *Glycyrrhiza uralensis* UGDs (GuUGDs), which were discovered to comprise five isoforms, four of which (GuUGD1–4) showed UGD activity in vitro. GuUGD1–4 had different biochemical properties, including their affinity for UDP-glucose, catalytic constant, and sensitivity to feedback inhibitors. GuUGD2 had the highest catalytic constant and highest gene expression level among the GuUGDs, suggesting that it is the major isoform contributing to the transition from UDP-glucose to UDP-glucuronic acid in planta. To evaluate the contribution of GuUGD isoforms to saponin biosynthesis, we compared the expression patterns of GuUGDs with those of saponin biosynthetic genes in methyl jasmonate (MeJA)-treated cultured stolons. GuUGD1–4 showed delayed responses to MeJA compared to those of saponin biosynthetic genes, suggesting that MeJA-responsive expression of GuUGDs compensates for the decreased UDP-glucuronic acid pool due to consumption during saponin biosynthesis.

Key words: *Glycyrrhiza uralensis*, triterpenoid saponin, UDP-glucose dehydrogenase, UDP-glucuronic acid.

Introduction

Plant cells have cell walls that consist mainly of cellulose, hemicellulose, and pectin. Unlike cellulose, which consists only of glucose (Delmer and Amor 1995), hemicellulose and pectin contain various sugar components, including glucose, galactose, rhamnose, glucuronic acid, galacturonic acid, xylose, arabinose, apiose, mannose, and fucose (Caffall and Mohnen 2009; Ebringerová et al. 2005; Mohnen 2008). The monomeric precursors of the sugars in these polysaccharides are two sugar nucleotides: uridine 5′-diphosphate (UDP) sugars and guanosine 5′-diphosphate (GDP) sugars. UDP sugars are commonly synthesized from UDP-glucose, which is available from photosynthesis assimilates. UDP-xylene, UDP-arabinose, UDP-apiose, and UDP-galacturonic acid are derived from UDP-glucuronic acid (Feingold 1982; Seifert 2004), which is synthesized from UDP-glucose via irreversible two-step oxidation with no release of intermediates by UDP-glucose dehydrogenase (UGD; Ge et al. 2004). UGD activity is often the lowest in extracts containing pathway enzymes and is inferred as rate limiting in the synthesis of cell wall precursors (Amino et al. 1985; Dalessandro and Northcote 1977). Many studies have examined the biological roles of UGD in the biosynthesis of cell wall polysaccharides. One biological study suggested the importance of UGDs in the sugar nucleotide oxidation pathway and polysaccharide synthesis based on the reductions in arabinose, xylose, and apiose in the cell wall of *ugd2,3* double mutant *Arabidopsis thaliana* plants, which exhibit a dwarf phenotype (Reboul et al. 2011). Moreover, a reduced sugar composition was detected only in UGD-A mutants, not in UGD-B mutants, in *Zea mays*, suggesting

Abbreviations: ABA, abscisic acid; ADP, adenosine 5′-diphosphate; bAS, β-amyrin synthase; CDSs, coding sequences; FPKM, fragments per kilobase of exon per million mapped reads; GDP, guanosine 5′-diphosphate; MeJA, methyl jasmonate; NAD+, nicotinamide adenine dinucleotide; NADP+, nicotinamide adenine dinucleotide phosphate; ORFs, open reading frames; qPCR, quantitative real-time PCR; RPKM, reads per kilobase of exon per million mapped sequence reads; TDP, thymidine 5′-diphosphate; UDP, uridine 5′-diphosphate; UGD, UDP-glucose dehydrogenase; USP, UDP-sugar pyrophosphorylase.

This article can be found at http://www.jspcmb.jp/

Published online June 12, 2021
that UGD-A has a more important role than UGD-B in UDP-glucuronic acid synthesis (Kärkönen et al. 2005). Several UGD isoforms are present in plants, although comparative biochemical and biological studies of these isoforms have been limited to Z. mays and A. thaliana (Kärkönen et al. 2005; Klinghammer and Tenhaken 2007; Reboul et al. 2011). Plants have an alternative pathway for the biosynthesis of UDP-glucuronic acid that involves inositol oxygenase (Kanter et al. 2005; Kotake et al. 2007; Loewus et al. 1962; Seitz et al. 2000). In this pathway, UDP-glucuronic acid is synthesized from α-D-glucuronic acid 1-phosphate by UDP-sugar pyrophosphorylase (USP). Unlike UGDs, generally one USP isoform is present in plants.

Apart from the biosynthesis of cell wall polysaccharides, UDP-glucuronic acid is also used as a sugar donor for the glycosylation of various plant specialized metabolites, including flavonoids and triterpenoids. Nevertheless, the contribution of UGDs in plant specialized metabolisms has not been well studied.

Legumes, the third largest family of angiosperms, biosynthesize various glucuronosylated metabolites. Soyasaponins, which are representative plant specialized metabolites of legumes, are classified as oleanane-type triterpenoid saponins and are commonly glucuronosylated at the C-3 position of the triterpenoid scaffold. Moreover, Glycyrrhiza species (licorice), which are major medicinal legumes, characteristically accumulate glycyrrhizin, another oleanane-type triterpenoid saponin with a characteristic sugar chain composed of two glucuronic acids, in their roots and stolons (Hayashi and Sudo 2009). This unique sugar moiety of glycyrrhizin, which is 150 times sweeter than sucrose (Kitagawa et al. 1993), contributes sweetness (Esaki et al. 1978; Kitagawa 2002). Hence, licorice seems to be a good target for studying the contribution of UGD to the supply of UDP-glucuronic acid for triterpenoid saponin biosynthesis.

Most enzymes that catalyze the biosynthesis of soyasaponins or glycyrrhizin have been characterized in licorice. Both soyasaponins and glycyrrhizin are derived from β-amyrin, one of the most commonly occurring triterpenes in plants, which is biosynthesized from 2,3-oxidosqualene by β-amyrin synthase (bAS; Augustin et al. 2011; Hayashi et al. 2001; Thimmappa et al. 2014). In soyasaponin biosynthesis, the two cytochrome P450 monoxygenases (P450) CYP93E3 and CYP72A566 catalyze oxidation reactions at the C-24 and C-22β positions of β-amyrin, respectively, to produce soyasapogenol B as an aglycone part of soyasaponin I and II (Seki et al. 2008; Tamura et al. 2018). Soyasaponin I and II are produced via three glycosylation steps, in which first glucuronic acid, then galactose or arabinose, and finally rhamnose are attached. In glycyrrhizin biosynthesis, the P450s CYP88D6 and CYP72A154 catalyze oxidation reactions at the C-11 and C-30 positions of β-amyrin, respectively, producing glycyrrhetinic acid (Seki et al. 2008, 2011). Finally, two-step glucuronosylation of glycyrrhetinic acid at the C-3 hydroxy group by two glucuronosyltransferases, a cellulose synthase-like enzyme GuCSyGT (GuCsl) and UGT73P12, produces glycyrrhizin via glycyrrhetinic acid 3-O-mono-glucuronide (Chung et al. 2020; Joziwiak et al. 2020; Nomura et al. 2019). However, no enzyme producing UDP-glucuronic acid has been identified in licorice.

To investigate the possible functional differentiation between UGD isoforms in specialized metabolisms in relation to cell wall component biosynthesis, UGDs should be identified and characterized individually. Therefore, in this study, we identified and characterized Glycyrrhiza uralensis UGDs (GuUGDs), which were discovered to have five isoforms. We analyzed the biochemical properties of each GuUDG isoform and examined their individual roles in the transition from UDP-glucose to UDP-glucuronic acid. We also explored the possible contribution of each GuUDG isoform to the sugar donor supply for triterpenoid saponin biosynthesis by comparing gene expression patterns between GuUGDs and known saponin biosynthetic genes.

**Materials and methods**

**Plant materials**

Roots of *G. uralensis* strain 308-19 harvested in June 2011 (Ramilowski et al. 2013) were used for RNA extraction to isolate UGD genes. Tissue-cultured stolons of *G. uralensis* (Hokkaido-iryodai strain) were maintained in Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands) supplemented with 6% sucrose and 0.01 mM 1-naphthaleneacetic acid (NAA), as reported previously (Kojoma et al. 2010). RNA was extracted from the roots of 3-week-old *Medicago truncatula* accession R108 grown in plant chambers under a 16-h-light/8-h-dark photoperiod at 24°C.

**Database searches and isolation of UGD genes**

We identified GuUGD unigene sequences with blastx similarity search against 32,840 protein coding sequences derived from *G. uralensis* transcriptome data (Ramilowski et al. 2013, http://ngs-data-archive.psc.riken.jp/Gur/index.pl) using the A. thaliana UGD1 gene sequence, previously isolated and characterized by Oka and Jigami (2006), as a query. To obtain the full-length coding sequences (CDSs) of six unigenes containing partial CDSs, we performed rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) using the SMARTer RACE cDNA Amplification Kit (Clontech/Takara Bio, Shiga, Japan). The full-length CDSs of *GuUGDs* were PCR-amplified from the first-strand cDNA library prepared from roots of *G. uralensis* strain 308-19 with primers 1–10 (Supplementary Table S1) and PrimeSTAR Max DNA Polymerase (Takara)
Bio), then cloned into pENTR/DTOPO (Thermo Fisher Scientific, Waltham, MA, USA) to produce an entry clone. After sequencing, each gene was assigned a *G. uralesis* gene ID (Supplementary Table S2) through a similarity search against the *G. uralesis* genome database (http://ngs-data-archive.psc.riken.jp/Gur-genome/index.pl). The nucleotide sequences isolated in this study have been submitted to the DNA Data Bank of Japan (DDBJ) under accession numbers LC528155 (GuUGD1), LC528156 (GuUGD2), LC528157 (GuUGD3), LC528158 (GuUGD4), and LC528159 (GuUGD5).

We identified putative *M. truncatula* UGD (MtUGD) protein sequences in a blastp similarity search against GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using the isolated GuUGD1–5 sequences as queries. The corresponding genes (MtUGD1–3) were isolated from cDNA derived from *M. truncatula* root RNA using primers 22–33 (Supplementary Table S3).

**Recombinant protein expression in Escherichia coli**

Full-length CDSs encoding the GuUGD and MtUGD isoforms and *A. thaliana* UGD2 (used as a positive control) were cloned from entry clones into the *Sac* I and *Sal* I sites of the His-tag expression vector pCOLD I DNA (TaKaRa Bio) with the In-Fusion HD cloning kit (TaKaRa Bio) and primers 11–21 (Supplementary Table S1). The coding sequence of *Arabidopsis* UGD2 was amplified from the cDNA clone RAFL09-33-I02 provided by the RIKEN BRC through the national Bio-Resource Project of the MEXT, Japan. The plasmid was introduced into *E. coli* strain BL21 Star (DE3; Invitrogen, Carlsbad, CA, USA) with the chaperone plasmid pGro7 (TaKaRa Bio). To obtain recombinant proteins, we grew bacterial cultures at 37°C to an optical density at 600 nm of approximately 0.4–0.8 in LB medium supplemented with 0.5 mg ml⁻¹ L-arabinose, 20 µg ml⁻¹ chloramphenicol, and 100 µg ml⁻¹ ampicillin. After cooling the cultures for 30 min at 15°C, we induced protein expression by adding 500 µM isopropyl β-D-thiogalactopyranoside (IPTG). After 1 day of cultivation at 15°C, cells were cooled by being shaken on ice, collected by centrifugation (4°C, 10 min, 9,500×g), and frozen in liquid nitrogen.

**Purification of the enzyme**

The frozen cells were resuspended in chilled disruption buffer (10 mM Tris, 50 mM sodium phosphate, 2 mM MgCl₂, 10% [v/v] glycerol, 1 mM NAD⁺, 2 mM 2-mercaptoethanol, and 1× Complete Protease Inhibitor Cocktail [Roche, Basel, Switzerland]; pH 8.0) at 10 µg ml⁻¹ fresh weight (FW). Lysozyme (200 µg ml⁻¹) and Nonidet P-40 (1%, v/v) were added to the suspension, and the mixture was shaken slowly at 4°C for 30 min. Then 2.4 U ml⁻¹ benzonase nuclease (Sigma-Aldrich, St. Louis, MO, USA) was added, followed by incubation for 15 min with slow shaking at 4°C. Bacterial debris was removed by centrifugation (10 min, 9,500×g), and the clear supernatant was applied to a TALON metal affinity resin column (Clontech) equilibrated with purification buffer (10 mM Tris, 50 mM sodium phosphate, 300 mM NaCl, and 10% glycerol; pH 8.0) after the addition of 250 mM NaCl. Extracts were purified with the batch/gravity-flow method. The column was washed twice with 10 volumes of purification buffer to wash out all unbound proteins. Weakly bound proteins were eluted from the TALON column with five volumes of purification buffer containing 10 mM imidazole. The UGD enzyme was eluted in purification buffer containing 100 mM imidazole and immediately concentrated in storage buffer (20 mM Tris, 50 mM KCl, 0.1 mM EDTA, 10% [v/v] glycerol, and 0.5 mM NAD⁺; pH 8.0) by ultrafiltration on an Amicon Ultra 100 k device (Millipore, Burlington, MA, USA). The recombinant enzyme was stored at −80°C. His-tagged UGD was detected by western blotting with Anti-His-tag mAb-HRP-DirecT (Medical & Biological Laboratories, Aichi, Japan) and Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan). The quality of the purified UGDs was verified by Coomassie Brilliant Blue (CBB) staining and UV detection after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentrations of the purified UGDs were calculated from the intensity of the corresponding band in the CBB-stained gel.

**Enzyme assays**

Enzyme activity of UGDs was analyzed by two approaches: analysis of reaction products with ultra-performance liquid chromatography–mass spectrometry (UPLC–MS); or analysis of the initial velocity by photometrical monitoring of the time-dependent increase in NADH converted from the cofactor NAD⁺, monitored at 340 nm with the Infinite 200 PRO multimode microplate reader (Tecan, Männedorf, Switzerland). All reactions were performed at 25°C in 100 µl of assay buffer (20 mM Tris, 1 mM EDTA, and 10% [v/v] glycerol; pH 8.7) following the addition of enzyme that had been preincubated for 90 min in storage buffer at room temperature. UDP-glucuronic acid production was confirmed by UPLC–MS using 1 µg of each isoform in a reaction solution containing 2 mM UDP-glucose and 600 µM NAD⁺ after determination of the optimal condition. To determine the optimal pH, 1 µg of GuUGD1–4 was analyzed at pH 4.0, 6.0, 8.0, and 10.0 to roughly determine the optimal pH range; then, GuUGD4 was analyzed using smaller increments between pH 7.1 and 10.6. We used glycine buffer for pH range 9.0–10.6 instead of the Tris buffer.

We evaluated the substrate specificity of each isoform by comparing the initial velocity with a standard enzyme assay containing 500 µM UDP-glucose and NAD⁺ as substrate and cofactor, respectively. The amounts of the enzymes were adjusted so that the conversion rate was in the range of 0.01 to 0.1 µM s⁻¹ in the standard enzyme assay. GuUGD5, for which the conversion rate could not be calculated, was used in this assay in the same amount as GuUGD4 (20-fold larger volumes than GuUGD4 solution). All sugar nucleotides and cofactors were used at a concentration of 500 µM.

To determine kinetic parameters, we used sufficient concentrations of NAD⁺ (1 mM) and various concentrations of
UDP-glucose (0–2,000μM), or various NAD⁺ concentrations (5–1,305μM) and a constant UDP-glucose concentration (2mM). The reaction was performed three times using 1μg of GuUGD1–4, and we calculated kinetic parameters from the nonlinear least squares based on the Gauss-Newton method using Python.

We evaluated the inhibitor sensitivity by comparing the initial velocity to the standard enzyme assay. The standard enzyme assay for inhibitor sensitivity was performed without an inhibitor, using UDP-glucose at twice the concentration of each Km calculated in the kinetic analyses. The amounts of the enzymes were adjusted so that the conversion rate was in the range of 0.01 to 0.1μM s⁻¹ without inhibitors.

**Reaction product analyses using UPLC–MS**

Reaction products were analyzed with an ACQUITY Ultra-Performance LC system with a tandem quadruple detector (Waters, Milford, MA, USA) using hydrophilic interaction chromatography on an ACQUITY UPLC BEH Amide column (1.7μm, 2.1×150mm; Waters) maintained at 75°C; 5μl of each sample was injected for analyses. The mobile phase consisted of 50:50 acetonitrile/water with 10mM triethylamine acetate. The flow rate was set at 0.4ml min⁻¹. Compounds were ionized by electrospray ionization in negative ion mode. Mass spectra were recorded in the range of 100–750m/z. The settings of the mass spectrometer were as follows: capillary voltage, +3.5kV; cone voltage, 45V; source temperature, 150°C; desolvation temperature, 450°C; cone gas flow, 50l h⁻¹; and desolvation gas flow, 450l h⁻¹. MassLynx (ver. 4.1; Waters) was used for data acquisition and analyses. We identified peaks by comparing their retention times with that of authentic standard UDP-glucuronic acid (Nacalai Tesque).

**3D Modeling**

The human UGD homo-hexamer structure (Beattie et al. 2018) was used as a template. 3D-structure models of GuUGDs were constructed using the automated SWISS-MODEL server at ExPaSy (https://swissmodel.expasy.org/; Benkert et al. 2011; Bertoni et al. 2017; Bienert et al. 2017; Guex et al. 2009; Waterhouse et al. 2018) based on protein homology. The predicted 3D structures of the GuUGDs were analyzed via superimposition on the X-ray crystal structure of human UGD with RasMol ver. 2.7.5.2.

**Treatment of tissue-cultured stolons with abscisic acid**

Tissue-cultured stolons were cultured for 2 weeks in MS medium supplemented with 6% sucrose without NAA before treatment. For abiotic stress treatments in time-course quantitative real-time PCR (qPCR) analyses, we diluted abscisic acid (ABA; BD Biosciences, San Jose, CA, USA) with ethanol to make 10mM stock solutions, and then added 1ml of stock solutions to 100ml of medium (final concentration, 100µM). Ethanol (1ml) was added to 100ml of medium as a mock treatment.

**RNA extraction and first-strand cDNA synthesis**

Total RNA was extracted from frozen tissue-cultured stolons with PureLink Plant RNA Reagent (Thermo Fisher Scientific), then treated with recombinant DNase I (RNase-free; Takara Bio) and purified with the RNaseasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. First-strand cDNA was synthesized with PrimeScript RT Master Mix (Perfect Real Time; Takara Bio) from 2.5μg total RNA in a 50-μl reaction.

**qPCR**

We mixed 1× FastStart Essential DNA Green Master (Roche), 500nM primers, and 0.5μl of cDNA, and the reaction volume was brought to 10μl with PCR-grade water. cDNA for the MeJA treatment analyses was synthesized from total RNA extracted from tissue-cultured stolons treated with 100µM MeJA or 0.1% ethanol (mock for MeJA), as described previously (Tamura et al. 2017). Reactions were performed with a LightCycler Nano (Roche) at 95°C for 10 min, followed by 45 cycles of 95°C for 10s, 60°C for 10s, and 72°C for 15s. The data were analyzed with LightCycler Nano ver. 1.1.0 (Roche). We calculated the relative transcript levels of each target gene using β-tubulin (Seki et al. 2008; GenBank accession number LC318135) as a reference gene. Each sample was amplified three times with primers 34–57 (Supplementary Table S4) designed using the Primer3 website (http://bioinfo.ut.ee/primer3-0.4.0/; Koressaar and Remm 2007; Untergasser et al. 2012).

**Bioinformatics and phylogenetic analyses**

We retrieved homologous plant UGD protein sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) by blastp search using the isolated GuUGD1–5 sequences as queries. Sequences containing irregularly long N- or C-termini were removed. Phylogenetic trees were generated by the maximum likelihood method based on the JTT matrix-based model (Jones et al. 1992) after alignment by ClustalW in MEGA X (Kumar et al. 2018). Homologous UGD genes were retrieved as reference sequences of each homologous protein. Genes located upstream and downstream of each UGD homolog in G. uralensis and other plants were retrieved from the G. uralensis genome database (http://ngs-data-archive.psc.riken.jp/Gur-genome/index.pl) and GenBank, respectively.

**Gene expression analyses of Fabales UGDs**

Gene expression data for UGD based on fragments per kilobase of exon per million mapped reads (FPKM; G. uralensis), reads per kilobase of exon per million mapped sequence reads (RPKM; Glycine max), or normalized expression in microarrays (M. truncatula) were retrieved from public transcriptome data for each putative UGD gene. Normalized data derived from RNA extracted from roots and leaves of G. uralensis collected in summer (G. uralensis database; http://ngs-data-archive.psc.riken.jp/Gur/index.pl, Ramilowski et al. 2013), from roots and young leaves of G. max (SoyBase; https://www.soybase.org/, Severin et al. 2010), and from roots and leaves of M. truncatula...
(Mt Gene Expression Atlas; https://mtgea.noble.org/v3/, Benedito et al. 2008; He et al. 2009) were analyzed for UGD expression. Because GuUGD3, GuUGD4, and GuUGD5 each corresponded to two partial unigene sequences (Supplementary Table S2), their FPKM values were recalculated as follows: expression of GuUGD=(raw reads of unigene I+raw reads of unigene II)/(raw reads of unigene I/FPKM of unigene I+raw reads of unigene II/FPKM of unigene II). The number correspondence between GenBank and each database is shown in Supplementary Table S5.

Results

Identification of UDP-glucose dehydrogenases in G. uralensis

To identify UGDs in G. uralensis, we performed a blastx search against 32,840 protein coding sequences derived from G. uralensis transcriptome data (Ramilowski et al. 2013) using the A. thaliana UGD1 gene sequence as a query. We found eight unigene sequences, including two complete and six partial open reading frames (ORFs), encoding putative GuUGDs. RACE-PCR amplification revealed that the six partial sequences were derived from three genes. Thus, complete ORFs of five putative GuUGDs (referred to as GuUGD1–5) were obtained and assigned to five independent gene IDs, located on different scaffolds, in the G. uralensis genome database (Mochida et al. 2017; Supplementary Table S2). The deduced amino acid sequences of GuUGD1–5 showed high amino acid sequence identity (79–86%) to A. thaliana UGD1 (Table 1). Furthermore, very high identities were found among GuUGD1–5 (81–93%; Table 1).

In vitro enzyme activity of recombinant UGDs

We successfully expressed GuUGDs in E. coli by cloning the ORF of each GuUGD into a cold-shock expression vector together with GroES/EL chaperones. The purified GuUGDs showed a protein band at approximately 50 kDa in a UV-illuminated SDS-PAGE gel and by His-tag detection in a western blotting gel (Figure 1A). The expression of recombinant GuUGD5 was much lower than that of the others, so GuUGD5 was purified at a low concentration and low purity (Figure 1A). The concentration of each purified GuUGD was calculated from the intensity of the corresponding band in CBB-stained gels, and UGD activity was analyzed using the same amount of purified GuUGD. Enzyme activity was detected using two approaches: detecting reaction products by UPLC–MS and photometrical monitoring of the time-dependent increases in NADH that was converted from the cofactor NAD⁺. Generally, the optimal pH of plant UGDs is 8.0–9.0 (Davies and Dickinson 1972; Hinterberg et al. 2002; Stewart and Copeland 1998; Strominger and Mapson 1957; Turner and Botha 2002). Consistently, increases in NADH were detected at pH 8.0 and 10.0, but not at pH 4.0 or

<table>
<thead>
<tr>
<th>GuUGD1</th>
<th>GuUGD2</th>
<th>GuUGD3</th>
<th>GuUGD4</th>
<th>GuUGD5</th>
<th>AtUGD1</th>
</tr>
</thead>
<tbody>
<tr>
<td>89%</td>
<td>90%</td>
<td>81%</td>
<td>86%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>93%</td>
<td>85%</td>
<td>83%</td>
<td>84%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>86%</td>
<td>83%</td>
<td>83%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>81%</td>
<td>83%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>79%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Figure 1. UGD activity of purified UGD recombinant proteins.](image-url)
Functional analyses of UGD isoforms in *G. uralensis*

6.0 using GuUGD1–4 in preliminary experiments. Further analysis revealed that the UGD enzyme activity level was high at pH 8.5–9.0 (Supplementary Figure S1). Because of the difficulty in obtaining large amounts of purified GuUGD5, we could not perform optimization for GuUGD5; thus, GuUGD5 was analyzed under the optimal conditions for the other GuUGDs.

As shown in Figure 1B, with the coexistence of UDP-glucose as a substrate and NAD⁺ as a cofactor, GuUGD1–4 and AtUGD2 (positive control) yielded a single reaction product that showed the same *m/z* (579.1) and retention time (0.74 min) as the authentic UDP-glucuronic acid standard. This peak was not detected in GuUGD5 products or the empty vector control (Figure 1B). Consistent with this result, an increase in NADH was detected in the reactions containing GuUGD1–4 in the photometrical analyses, whereas it was not detected in the GuUGD5-containing reaction (Table 2). Some plant UGDs accept other sugar nucleotides as minor substrates and NADP⁺ as a minor cofactor with weak catalytic activity (0.5–23% of the main activity; Klinghammer and Tenhaken 2007; Stewart and Copeland 1998; Turner and Botha 2002). Hence, we analyzed enzyme activity for other substrates (UDP-galactose, UDP-N-acetylglucosamine, TDP-glucose, ADP-glucose, and GDP-glucose) in the presence of either NAD⁺ or NADP⁺. In the presence of NAD⁺, only GuUGD4 accepted UDP-galactose and UDP-N-acetylglucosamine as minor substrates with 15% and 10% activity compared to UDP-glucose, respectively (Table 2). By contrast, GuUGD5 did not show enzyme activity for any substrate. None of the GuUGDs used NADP⁺ as a cofactor instead of NAD⁺.

To obtain deeper insight into the biochemical properties of each active GuUGD isoform, we analyzed kinetic parameters for UDP-glucose and NAD⁺. GuUGD1–4 exhibited typical hyperbolic reaction kinetics and were not inhibited by higher substrate concentrations (Supplementary Figure S2). Affinity for the substrate UDP-glucose and the cofactor NAD⁺ differed among the GuUGD isoforms (Table 3). GuUGD4 showed the highest affinity for UDP-glucose, with approximately less than one-fifth of the *Kₘ* of the other active GuUGD isoforms. The highest catalytic

### Table 2. Substrate specificity of GuUGDs.

<table>
<thead>
<tr>
<th>Nucleotide-sugar</th>
<th>GuUGD1</th>
<th>GuUGD2</th>
<th>GuUGD3</th>
<th>GuUGD4</th>
<th>GuUGD5</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-glucose</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

We calculated enzyme activity based on initial velocity within 10 min measured by monitoring the conversion of NAD(P)⁺ to NAD(P)H, detected by the absorbance at 340 nm. 100% corresponds to the activity indicated in the standard assay with UDP-glucose as a substrate and NAD⁺ as a cofactor. n.d., not detected.

### Table 3. Kinetic parameters of GuUGDs.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Kinetics for UDP-glucose</th>
<th>Kinetics for NAD⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Kₘ</em> (µM)</td>
<td><em>kₐₚ</em> (s⁻¹)</td>
</tr>
<tr>
<td>GuUGD1</td>
<td>261±84</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>GuUGD2</td>
<td>285±54</td>
<td>0.89±0.09</td>
</tr>
<tr>
<td>GuUGD3</td>
<td>570±46</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>GuUGD4</td>
<td>55±5</td>
<td>0.25±0.02</td>
</tr>
</tbody>
</table>

We calculated enzyme activity based on initial velocity within 5 min measured by monitoring the conversion of NAD⁺ to NADH, detected by the absorbance at 340 nm. Values represent the mean±SE of three measurements.

### Table 4. Inhibitory effects of UDP-xylose and UDP-glucuronic acid on GuUGD1–4.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Relative enzyme activity of isoforms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GuUGD1</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>UDP-xylose (20µM)</td>
<td>80</td>
</tr>
<tr>
<td>UDP-xylose (500µM)</td>
<td>n.d.</td>
</tr>
<tr>
<td>UDP-glucuronic acid (20µM)</td>
<td>104</td>
</tr>
<tr>
<td>UDP-glucuronic acid (1250µM)</td>
<td>95</td>
</tr>
</tbody>
</table>

Enzyme activity was calculated based on initial velocity within 10 min by monitoring the conversion of NAD⁺ to NADH, detected by the absorbance at 340 nm. All inhibitors were added just before the reaction was initiated. The concentration of the substrate UDP-glucose was twice the concentration of each *Kₘ* calculated in the kinetic analyses. 100% corresponds to the activity indicated in the standard assay performed without an inhibitor. n.d., not detected.
constant was detected in GuUGD2, with more than twice the $k_{cat}$ of the other active GuUGD isoforms.

Fine tuning of UGD activity is mediated by feedback inhibition of the enzyme by UDP-xylose, a product obtained from UDP-glucuronic acid after decarboxylation by UDP-xylose synthase (Hinterberg et al. 2002; Neufeld and Hall 1965; Turner and Botha 2002). The enzyme activity of all GuUGD isoforms was strongly inhibited and could scarcely be detected in the presence of 500 $\mu$M UDP-xylose (Table 4). The inhibitory effect differed at low UDP-xylose concentrations. GuUGD4 was the most sensitively inhibited by UDP-xylose at 20 $\mu$M, while GuUGD3 was not inhibited. Unlike UDP-xylose, UDP-glucuronic acid showed very weak inhibitory effects, as seen in other plants (Davies and Dickinson 1972; Oka and Jigami 2006; Turner and Botha 2002). Only GuUGD4 was sensitive to UDP-glucuronic acid. GuUGD1, GuUGD2, and GuUGD3 were not inhibited, even by high concentrations of UDP-glucuronic acid.

**Molecular modeling and structural analyses of UGDS**

In general, UGD amino acid sequences share striking identity among plants, animals, and microorganisms: amino acid sequences of *A. thaliana* UGD1 have 85% identity to *G. max* UGD, 58% identity to human UGD, and 22% identity to *Streptococcus pyogenes* UGD. As shown in Table 1, the five GuUGD isoforms showed very high amino acid sequence identities (81–93%) to one another; however, GuUGD5 showed no enzyme activity in this study. To explore the key amino acid residues affecting the activity of GuUGDs, we performed amino acid sequence comparison of the GuUGD isoforms. The five GuUGDs, comprising 480–482 amino acids, contained the full-length functional catalytic motifs (NAD$^+$ binding motif, UDP binding motif, and central motif) and several residues were found to be specific to GuUGD5 among the five GuUGDs (Figure 2A). Several residues have been proposed as important residues for catalysis (shown in asterisks); some residues were proposed based on bond distances in native and mutant *S. pyogenes* UGD (Campbell et al. 2000), and some residues were proposed based on residue occupancy within the binding site and comparative kinetic analyses of wild-type and mutated human UGD (Egger et al. 2011). Several residues in human UGD were also identified as key residues that have hydrophobic interactions, hydrogen bonds, salt bridges, and water bridges with UDP-glucose (shown in circles) and NAD$^+$ (shown in squares) in SWISS-MODEL. Among the residues specific to GuUGD5, Lys257 replaced arginine, which positions a key residue with a hydrogen bond in human UGD, with UDP-glucose in SWISS-MODEL (red box, Figure 2A). To confirm that this residue is located near UDP-glucose in the GuUGDs, we constructed homology-based structural models of the GuUGDs using human UGD (Beattie et al. 2018) as a template. The 3D structural models of GuUGD2 and GuUGD5, which displayed high sequence identity (59.4–61.5%) to human UGD (Supplementary Table S6), were predicted by SWISS-MODEL using human UGD as template. The 3D structural models of GuUGD1, GuUGD3, and GuUGD4 could not be predicted with this crystal structure in SWISS-MODEL and thus were predicted with the constructed 3D structure of GuUGD2 as a template. Superimposition analyses of the crystal structure of human UGD with the GuUGD models showed a highly conserved structure in all enzymes (Figure 2B).

**Expression patterns of GuUGD genes**

The gene expression of each GuUGD isoform was compared based on FPKM values obtained in RNA-Seq analyses of *G. uralensis* plants (Ramilowski et al. 2013). As shown in Figure 3A, GuUGD2 had the highest expression among all GuUGD genes in both roots and leaves. GuUGD1 showed moderate expression, whereas GuUGD3 and GuUGD4 showed weaker expression. GuUGD5 was expressed weakly in both roots and leaves.

UGD expression is upregulated in leaves of *Populus tomentosa* under various stressful conditions, including ABA treatment (Tian et al. 2014). In *G. uralensis*, the accumulation of glycyrrhizin was promoted by exogenous ABA (Qiao et al. 2017). To obtain additional insight into the biological roles of each active GuUGD isoform, we analyzed the gene expression responses to ABA treatment in tissue-cultured stolons. Only GuUGD4 showed a strong expression response to ABA treatment (Figure 3B). GuUGD1 and GuUGD2 showed quite small responses to ABA treatment, while GuUGD3 was barely affected (Figure 3B). GuUGD5 did not differ significantly between ABA and mock treatments (data not shown).

**Transcriptional response of GuUGD genes to MeJA compared to saponin biosynthetic genes**

The biosynthesis of glycyrrhizin in the roots and hairy roots of *Glycyrrhiza* species is upregulated following the application of exogenous MeJA (Shabani et al. 2009; Wongwicha et al. 2011). Similarly, the biosynthesis of soyasaponin responds to MeJA treatment in cultured cells and tissue-cultured stolons (Hayashi et al. 2003; Tamura et al. 2018). To evaluate the possible involvement of each active GuUGD isoform in glycyrrhizin and
soyasaponin biosynthesis, we analyzed the effects of MeJA on the expression of \textit{GuUGD1–4}, as well as on known saponin biosynthetic genes, in tissue-cultured stolons of \textit{G. uralensis}. \textit{GuUGD1–4} expression was induced after MeJA treatment (Figure 4). The expression of \textit{GuUGD} genes was strongly enhanced after 6h
(peak times 12 or 48 h), with the greatest enhancement seen in GuUGD4. However, the upregulation of GuUGD gene expression was late compared to that of the glucuronosyltransferase genes CSyGT and UGT73P12. CSyGT and UGT73P12 expression were already upregulated at 3 h (peak time 6 h). Soyasaponin biosynthetic genes (bAS, CYP93E3, CYP72A566 and CSyGT) showed very similar expression responses to one another, with strong upregulation after 3 h. By contrast, glycyrrhizin biosynthetic genes (bAS, CYP88D6, CYP72A154, CSyGT, and UGT73P12) showed different response times to MeJA treatment. Upregulation of CYP88D6 expression immediately subsided at 6 h, although the enhanced bAS gene expression continued to strengthen, and the enhancement of CYP72A154 gene expression did not reach a peak by this time.

**Discussion**

We identified four active UGD isoforms (GuUGD1–4) in *G. uralensis*. Another isoform (GuUGD5) was also analyzed; however, it showed no enzyme activity for any substrate tested in this study. The affinity of GuUGD1–4 for UDP-glucose and NAD\(^+\) was profiled as *K_m* values of 55–570 \(\mu\)M and 31–153 \(\mu\)M, respectively, which is consistent with UGDs characterized in other plants: the affinity of recombinant UGDs has been reported as *K_m* values of 15–335 \(\mu\)M and 67–70 \(\mu\)M for UDP-glucose and NAD\(^+\), respectively, in soybean (Hinterberg et al. 2002), *A. thaliana* (Klinghammer and Tenhaken 2007; Oka and Jigami 2006), and *Eucalyptus grandis* (Labate et al. 2010), and the affinity of UGDs purified from plant tissues has been reported as *K_m* values of 19–950 \(\mu\)M and 72–400 \(\mu\)M for UDP-glucose and NAD\(^+\), respectively, in pea (Strominger and Mapson 1957), lily (Davies and Dickinson 1972), wheat (Stewart and Copeland 1998), canola (Stewart and Copeland 1998), soybean (Stewart and Copeland 1998), sugarcane (Turner and Botha 2002), and maize (Kärkönen et al. 2005). The calculated catalytic constants of GuUGD1–4 were 0.1–1.0 s\(^{-1}\), which is lower than those of other plants: catalytic activity of UGD has been reported as *k_cat* values of 1.17–2.52 s\(^{-1}\) in *A. thaliana* recombinant UGDs (Klinghammer and Tenhaken 2007) or *V_max* values of 2.17 and 68–172 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) in sugarcane UGD purified from plant tissues (Turner and Botha 2002) and *E. grandis* recombinant UGD (Labate et al. 2010; it can be calculated as 1.8–143 s\(^{-1}\) because UGDs were reported as ~50 kDa), respectively. GuUGD2 had the highest catalytic constant (1.0 s\(^{-1}\)), similar to that of AtUGDs (1.17–2.52 s\(^{-1}\)), with 2- to 10-fold higher *k_cat* values than the other active GuUGD isoforms (Table 3). GuUGD2 also had the highest gene expression level among the GuUGDs (Figure 3A), which suggests that GuUGD2 is the major isoform contributing to the transition from UDP-glucose to UDP-glucuronic acid in planta.

GuUGD4 showed the highest affinity for UDP-glucose, which suggests that GuUGD4 can produce UDP-glucuronic acid under various conditions, including UDP-glucose deficiency. Meanwhile, GuUGD4 showed the highest sensitivity for feedback inhibition to 20 \(\mu\)M UDP-xylose and UDP-glucuronic acid, which suggests its inactivation under conditions in which abundant products are present. Due to the properties of GuUGD4, we would expect a minimum transition from UDP-glucose into UDP-glucuronic acid to be maintained by continuous catalysis by GuUGD4 unless it is inhibited by accumulated products. The exact concentration of UDP-glucose in *G. uralensis* is unknown; however, the concentration of UDP-glucose can be estimated to range from 15 \(\mu\)M to 3.5 mM for the following reasons. UDP-glucose has been detected in amounts of 0.007–1.6 nmol mg\(^{-1}\) FW in several...
Figure 4. MeJA-responsive expression of GuUGDs and saponin biosynthetic genes. Biosynthetic pathways of glycyrrhizin and soyasaponin were predicted in previous studies (Seki et al. 2008, 2011). Transcript levels were analyzed by qPCR. Transcript levels in the mock treatment tissue-cultured stolons are indicated by outlined bars; MeJA treatments are indicated by filled bars. Relative transcript levels 0 h after treatment were set equal to 1. Error bars indicate the SD of three technical replicates. Asterisks indicate significant differences in gene expression level between the MeJA and mock treatments at each time point. Student’s t test, *** p < 0.001, ** p < 0.01, * p < 0.05. Part of this figure was reproduced and modified from Plant and Cell Physiology 59(4) with permission from Oxford University Press.
Aside from its enzymatic properties, the total UGD activity can also be regulated by the differential expression of \textit{UGD} genes. Among the \textit{GuUGDs}, \textit{GuUGD4} was most strongly upregulated by ABA and MeJA, whereas the other \textit{GuUGDs}, including the putative major contributing isoform \textit{GuUGD2}, responded weakly to ABA or MeJA treatment compared to \textit{GuUGD4}. This strong response to stress hormones suggests a large contribution by \textit{GuUGD4} to the transition from UDP-glucose to UDP-glucuronic acid under these stressful conditions.

Plants often contain several UGD isoforms, probably generated via gene duplication. To reveal the molecular evolution of plant UGDs, we constructed a phylogenetic tree with various plant UGDs. As shown in Figure 5A, the phylogenetic tree contains three clades (gray backgrounds) composed of evenly assigned eudicot UGDs. No monocot UGDs belonged to these clades. This phylogenetic tree suggests that a gene duplication event occurred in the common ancestor of eudicots. In addition, sister clades of Fabales UGDs (clusters I & II and clusters III & IV) suggest an additional gene duplication in the common ancestor of Fabales.
Consistently, many legumes have five types of UGDs with conserved synteny (Supplementary Figure S3). Additionally, similar tissue-specific gene expression was observed for UGDs belonging to the same clusters (Figure 3A, Supplementary Figure S4). These observations suggest that characteristic biological functions are maintained within each cluster.

In the enzyme assays, GuUGD5 did not show UGD activity. Because of the low concentration and low purity of recombinant GuUGD5 due to difficulties expressing GuUGD5 in E. coli at levels as high as with GuUGD1–4 (Figure 1A), we used a large volume of protein solution to add the same amount of GuUGD5 as the other GuUGDs to the reaction mixture. Therefore, GuUGD5 might not have shown UGD activity because contaminating endogenous E. coli proteins prevented GuUGD5 activity. Other possible reasons are that GuUGD5 has no or very weak UGD activity lower than the detection limit. To obtain deeper insight into the biochemical properties of legume UGD cluster I–V, we analyzed MtUGDs. Four MtUGD sequences were found in a blastp search, and three of them were isolated (Supplementary Tables S5, S7). MtUGD1, MtUGD2, and MtUGD3 belong to clusters II, III, and V, respectively (Supplementary Figure S3). The MtUGDs were successfully expressed in E. coli and purified (Supplementary Figure S5A). In the enzyme assays, the highest peak intensity of UDP-glucuronic acid was seen with MtUGD2, which belongs to cluster III with GuUGD2 (Supplementary Figure S5B). On the other hand, the very small UDP-glucuronic acid peak detected in the MtUGD3 product suggests that UGDs belonging to cluster V have the weakest UGD activity in the five Fabales UGD clusters.

Among five GuUGDs, the amino acid substitution from Arg257 to Lys257, which likely decreases attraction to UDP-glucose, was found only in GuUGD5, while Arg257 was conserved in all characterized UGDs (Figure 5B). Interestingly, Lys257 was conserved in all cluster V UGDs (Supplementary Figure S6). Considered with the results of the enzyme assay, UGD activity might be weakened by the substitution from Arg257 to Lys257. Likewise, several amino acids were conserved in GuUGD and MtUGD belonging to the same cluster (Supplementary Figure S7). Cluster-specific biochemical properties are likely derived from these cluster-specific amino acids. To reveal the relation between biochemical properties and amino acid residues, further analysis is needed.

Soyasaponin biosynthetic genes, which are collectively regulated by the transcription factor GubHLH3 (Tamura et al. 2018), showed very similar expression patterns under MeJA treatment in tissue-cultured stolons. Expression of the GuUGD1–4 genes was enhanced under MeJA treatment, although the enhancement of GuUGD expression was delayed compared to that of soyasaponin and glycyrrhizin biosynthetic genes. These expression patterns seem to reflect the fact that GuUGD gene expression is regulated separately from that of triterpenoid saponin biosynthetic genes. We infer that MeJA-responsive expression of GuUGDs compensates for the decreased UDP-glucuronic acid pool due to consumption in saponin biosynthesis rather than prior or concomitant supply of UDP-glucuronic acid. Expression of a gene encoding a protein homologous to USP, which produces UDP-glucuronic acid from α-D-glucuronic acid 1-phosphate in the myoinositol pathway, was not upregulated ahead of the enhancement of glucuronosyltransferase gene expression (Supplementary Figure S8), which suggests that the alternative pathway was not used to supply UDP-glucuronic acid for saponin biosynthesis.

Overall, the results of this study suggest that no GuUGD isoforms exhibit functional differentiation that is specific to saponin biosynthesis. Genes in triterpene pathways are organized in operon-like clusters in some plant genomes to facilitate co-regulation of biosynthetic genes (Field and Osbourn 2008; Field et al. 2011; Kliebenstein and Osbourn 2012; Krokida et al. 2013; Nützmann and Osbourn 2014; Qi et al. 2004). However, no genes related to triterpenoid saponin biosynthesis were found around legume UGD genes (Supplementary Figure S3), which supports the idea that a UGD isoform specific to saponin biosynthesis did not form.

Acknowledgements

We are very grateful to the Takeda Garden for Medicinal Plant Conservation, Kyoto, Japan (Takeda Pharmaceutical Company Ltd.) for providing G. uralensis 308-19 strain plants. We are also very grateful to Dr. Kojima (Health Sciences University of Hokkaido) for providing tissue-cultured stolons of G. uralensis Hokkaido-iryodai strain. We thank Mr. Naoki Chiyo for technical advice and critical reading of the manuscript, and Ms. Keiko Fukamoto (Osaka University) for technical assistance.

References

Benkert P, Biasini M, Schwede T (2011) Toward the estimation
Labate MTV, Bertolo ALF, do Nascimento DD, Gutmanis G, De Andrade A, Rodrigues MJC, Camargo ELO, Boaretto LF, Moon...


