

Identification of a Highly Specific Isoflavone 7-O-glucosyltransferase in the soybean (*Glycine max* (L.) Merr.)

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The nucleotide sequences of *GmUGT* genes reported in this paper have been submitted to the DDBJ database under the following accession numbers: AB904891, *GmUGT2*; AB904892, *GmUGT3*; AB904893, *GmUGT4*; AB904894, *GmUGT7*; AB904895, *GmUGT8*; and, AB904896, *GmUGT9*.

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Isoflavone conjugates [7-O-β-D-glucosides and 7-O-(6''-malonyl-β-D-glucosides) of daidzein and genistein] accumulate in soybean roots and serve as the stored precursors of isoflavones (aglycons), which play very important roles in the rhizobia-mediated nodulation of this plant. Thus far, the isoflavone 7-O-glucosyltransferase (*GmIF7GT* or *GmUGT1*) has been biochemically characterized and is believed to be involved in isoflavone conjugate biosynthesis. The soybean genome encodes many other glycosyltransferase homologs (*GmUGTs*) that are related to *GmUGT1*; however, their catalytic properties, substrate specificities, and role(s) in isoflavone conjugation are unknown. In this study, nine different *GmUGT1*-related *GmUGT* cDNAs were isolated; six of these cDNAs belonged to two distinct phylogenetic subgroups (A and B), and these six were functionally characterized. The results showed that *GmUGT4*, a representative of subgroup A, encoded a UGT that was highly specific for isoflavones showing k_{cat} and k_{cat}/K_m values for daidzein of $5.89 \pm 0.65 \text{ s}^{-1}$ and $2.91 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$, respectively. Moreover, *GmUGT4* was expressed in the roots (mainly in lateral roots) of the 7-day-old seedlings and seeds, both of which contained abundant amounts of isoflavone conjugates. By contrast, *GmUGT1* and *GmUGT7*, which were subgroup B members, encoded enzymes with broad glucosyl-acceptor specificities and were mainly expressed in the aerial portions (cotyledons and hypocotyls) of the seedlings. In the present study, we proposed that the role of isoflavone glucosylation in a soybean plant is assigned to different *GmUGT* members in an organ/tissue-dependent manner. We also established the functional importance of *GmUGT4* in the biosynthesis of isoflavone conjugates in lateral roots that make a major contribution to overall N_2 fixation.

Keywords: Glucosyltransferase • *Glycine max* (L.) Merr. • Isoflavone • Lateral roots • Soybean.

Abbreviations: HPLC, high-performance liquid chromatography; Fw, fresh weight; UGT, UDP-sugar-dependent glycosyltransferase.

Introduction

Isoflavones are a class of flavonoids that are predominantly found in legumes, where they play important roles in plant-microbe interactions. In the soybean (*Glycine max* (L.) Merr.), isoflavones are excreted from roots and serve as signaling molecules in root nodulation (Dakora and Phillips 1996, Hungria and Stacey 1997). Isoflavones also play crucial roles inside the plant roots during legume nodulation (Subramanian et al. 2007). Isoflavones are also involved in the defense mechanisms of soybeans against infection from pathogens (Kramer et al. 1984, Graham et al. 1990, Dakora and Phillips 1996, Graham and Graham 2000).

In soybean roots, isoflavone aglycons (mainly daidzein) undergo an enzymatic 7-O-glucosylation and a subsequent 6''-O-malonylation (Suzuki et al. 2007) to produce isoflavone conjugates, which then accumulate in vacuoles (Barz and Welle 1992). The biological activities of isoflavones are considered to be dependent on the presence or absence of the conjugation (Pueppke et al. 1998, Smit et al. 1992). Thus, the conjugation of isoflavones is important for controlling the interactions of legumes with their symbiotic and pathogenic microorganisms. The first step in isoflavone conjugation is 7-O-glucosylation, which is specifically catalyzed by UDP-glucose:isoflavone 7-O-glucosyltransferase (Fig. 1). We previously reported the 14,000-fold purification of a UDP-sugar-dependent glycosyltransferase (UGT) from the roots of *G. max* seedlings (previously referred to as *GmIF7GT*, but referred to here as *GmUGT1*) (Noguchi et al. 2007), which efficiently catalyzes regiospecific glucosyl transfer to isoflavones at their 7-position.

The soybean genome (Schmutz et al. 2010) encodes a large number of UGT homologs (*GmUGTs*) that in phylogenetics are closely related to *GmUGT1*. In 2011, comprehensive transcription analyses of six of these homologs were carried out, and the tissue specificities of their transcriptions were reported (Livingstone et al. 2011). The results of these analyses showed that transcripts of another UGT homolog were as abundant as those of *GmUGT1*, and these were higher in the roots than those

of other homologs. However, the specificity and biochemical properties of this UGT remain to be examined. Hence, its role in flavonoid biosynthesis in the roots remains to be established.

In the present study, nine different *GmUGT* cDNAs that were related to *GmUGT1* were obtained, some of which could be classified into two phylogenetically different subgroups (A and B). Functional characterization of these cDNAs showed that *GmUGT4* (a representative of subgroup A) encodes a UGT that is highly specific for isoflavones and is mainly expressed in the lateral roots and seeds, while *GmUGT1* (a representative of subgroup B) encodes an enzyme displaying a broad glucosyl-acceptor specificity and is expressed in the aerial parts (cotyledons, hypocotyls and pods). Thus, in the soybean plant, the role of isoflavone glucosylation is assigned to different *GmUGT* members in an organ/tissue-dependent manner. The functional importance of *GmUGT4* in the biosynthesis of isoflavone conjugates in lateral roots has been discussed in conjunction with the proposed role of the stored precursors of isoflavones during rhizobia-mediated nodulation (Graham 1991).

Results

Cloning and expression of *GmUGT* homologs

In the soybean genome (Schmutz et al. 2010), we identified three new *GmUGT* genes (*GmUGT7*, *GmUGT8* and *GmUGT9*)

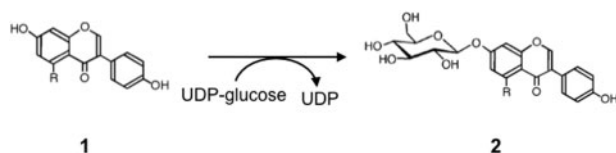


Fig. 1 Isoflavone 7-O-glucosyltransferase-catalyzed reaction (1: R=H, daidzein; R=OH, genistein. 2: R=H, daidzin; R=OH, genistin).

with sequence identities that were appreciably similar to that of *GmUGT1*. These genes are in addition to those identified previously by genomic analysis (*GmUGT2–GmUGT6*) (Livingstone et al. 2011) (**Table 1**). *GmUGT1* through *GmUGT8*, with the noted exception of *GmUGT6*, are located on chromosome 16. *GmUGT6* is located on chromosome 6, and *GmUGT9* resides on chromosome 9 (see **Supplemental Fig. S1**). A phylogenetic tree constructed for these *GmUGTs* on the basis of their amino acid sequences by means of the neighbor-joining method (**Fig. 2**) revealed that each of these *GmUGTs* was related to the UGT88 subfamily, as expected from their high sequence identities (greater than 40%, Yonekura-Sakakibara and Hanada 2011) with UGT88E3 (*GmUGT1*). Analysis clearly showed that *GmUGT2*, *GmUGT3*, *GmUGT4* and *GmUGT9* formed one phylogenetic subgroup (termed subgroup A), and that *GmUGT1*, *GmUGT7* and *GmUGT8* formed another subgroup (subgroup B). *GmUGT5* and *GmUGT6* were not assigned to these subgroups.

Because it was reported that *GmUGT2*, *GmUGT5* and *GmUGT6* were expressed at only negligibly low levels in soybean plants (Livingstone et al. 2011) and we confirmed this observation (data not shown), these homologs were not subjected to further activity studies. Other *GmUGTs* (*GmUGT1*, *GmUGT3*, *GmUGT4*, *GmUGT7*, *GmUGT8* and *GmUGT9*) are heterologously expressed in *Escherichia coli* cells as His₆-tagged proteins. The expressed products were purified to near homogeneity by nickel-affinity chromatography (**Supplementary Fig. S2**), with the exception of *GmUGT8*, which was obtained as an insoluble inclusion body. The committee responsible for UGT nomenclature (<http://www.flinders.edu.au/medicine/sites/clinical-pharmacology/ugt-homepage.cfm>) assigned these *GmUGTs* as follows: *GmUGT1*, UGT88E3; *GmUGT2*, UGT88E13; *GmUGT3*, UGT88E14; *GmUGT4*, UGT88E18; *GmUGT7*, UGT88E16; *GmUGT8*, UGT88E17; and *GmUGT9*, UGT88E15.

Table 1 Sequence identities (%) of *GmUGT* cDNAs are highlighted with a blue background and those of *GmUGT* proteins are highlighted with a gray background.

	<i>GmUGT1</i>	<i>GmUGT2</i>	<i>GmUGT3</i>	<i>GmUGT4</i>	<i>GmUGT5</i>	<i>GmUGT6</i>	<i>GmUGT7</i>	<i>GmUGT8</i>	<i>GmUGT9</i>
<i>GmUGT1</i>		63.3	64.0	63.3	45.7	41.7	97.4	78.6	63.6
<i>GmUGT2</i>	73.4		87.2	89.6	46.0	40.8	63.3	59.6	68.9
<i>GmUGT3</i>	73.9	88.8		88.5	47.4	40.4	63.5	58.8	67.0
<i>GmUGT4</i>	73.9	91.2	90.9		46.0	41.0	63.3	59.7	70.1
<i>GmUGT5</i>	59.9	55.9	58.6	58.9		45.2	45.8	45.3	48.2
<i>GmUGT6</i>	56.2	54.9	56.4	58.2	57.4		41.3	39.9	40.5
<i>GmUGT7</i>	98.5	73.3	73.9	73.9	60.1	56.9		78.6	63.1
<i>GmUGT8</i>	84.7	70.4	71.1	72.2	60.7	56.1	84.9		62.8
<i>GmUGT9</i>	72.9	74.9	75.7	76.7	60.5	57.0	73.1	73.1	

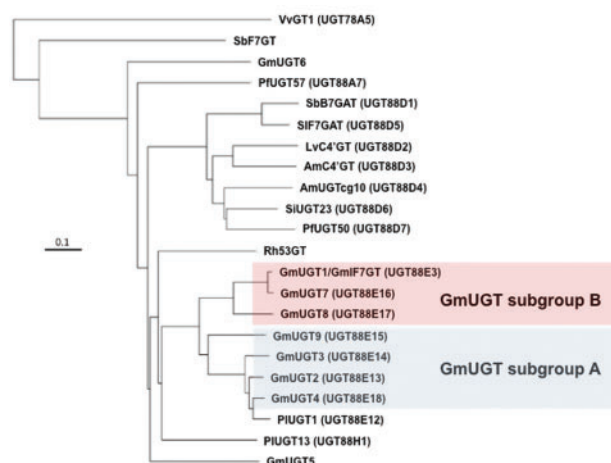


Fig. 2 Rooted phylogenetic tree of GmUGTs and UGT88-related PSPGs. The tree was constructed from a CLUSTALW program multiple alignment using a neighbor joining method. Bar = 0.1 amino acid substitutions/site. Names and DDBJ/EMBL/Genbank accession numbers of UGTs used for the alignment (other than GmUGTs) are as follows: AmC4'GT: *Antirrhinum majus* UDP-glucose:chalcone 4'-O-glucosyltransferase (accession number, AB198665); AmUGTcg10, *A. majus* UDP-glucuronic acid:flavonoid 7-O-glucuronosyltransferase (accession number, AB362988); LvC4'GT, *Linaria vulgaris* UDP-glucose: chalcone 4'-O-glucosyltransferase (accession number, BAE48240); PfUGT50, *Perilla frutescens* UDP-glucuronic acid:flavonoid 7-O-glucuronosyltransferase (accession number, AB362991); PfUGT57, *P. frutescens* UDP-glucose:flavonoid 7-O-glucosyltransferase (accession number, AB362992); PIUGT1, *Pueraria lobata* isoflavone 7-O-glucosyltransferase 1 (accession number, KC473565); PIUGT13, *P. lobata* isoflavone 7-O-glucosyltransferase 13 (accession number, KC473565); RhA53GT, *Rosa hybrida* UDP-glucose:anthocyanidin 5,3-O-glucosyltransferase (accession number, AB201048/BAD99560.1); SbB7GAT (accession number, AB362959), *Scutellaria baicalensis* UDP-glucuronic acid:baicalein 7-O-glucuronosyltransferase (accession number, AB479151); SbF7GT, *S. baicalensis* UDP-glucose:flavonoid 7-O-glucosyltransferase (accession number, BAA83484); SiUGT23, *Sesamum indicum* UDP-glucuronic acid:flavonoid 7-O-glucuronosyltransferase (accession number, AB362990); SIF7GAT, *Scutellaria laeteviolacea* var. *yakusimensis* UDP-glucuronic acid:flavonoid 7-O-glucuronosyltransferase (accession number, AB362989); and VvGT1, *Vitis vinifera* UDP-glucose:anthocyanin 3-O-glucosyltransferase (cv. Shiraz) (accession number, AAB81683).

Specificity and kinetic properties

The glucosyl-acceptor specificity of the purified GmUGTs was examined with a variety of flavonoids [genistein and daidzein (isoflavones), kaempferol and quercetin (flavonols), naringenin (a flavanone), and apigenin (a flavone)] using UDP-glucose as the glycosyl donor (Fig. 3; see Supplementary Fig. S3 for chemical structures). The enzymes of subgroup A, GmUGT3, GmUGT4 and GmUGT9, all were highly specific for isoflavones. Reactions of these GmUGTs with genistein and daidzein yielded single transfer products, each of which was co-eluted with genistin (genistein 7-O- β -D-glucoside) and daidzin (daidzein 7-O- β -D-glucoside), during analytical reversed-phase HPLC. The specific activities for genistein and daidzein were, respectively, 3.48 ± 0.23 nkat/mg and 4.22 ± 0.37 nkat/mg (for GmUGT3),

4.23 ± 0.37 nkat/mg and 7.78 ± 0.80 nkat/mg (for GmUGT4) and 0.75 ± 0.03 nkat/mg and 0.82 ± 0.05 nkat/mg (for GmUGT9). The flavonols (kaempferol and quercetin), flavanone (naringenin), flavones (apigenin and luteolin) and equol were all inert as substrates for these enzymes.

Although GmUGT1, a well-known enzyme (Noguchi et al. 2007), displayed high specific activities toward isoflavones (7.75 ± 0.35 nkat/mg for genistein and 7.26 ± 0.73 nkat/mg for daidzein) to produce their 7-O- β -D-glucosides, it also showed considerable levels of activities toward other flavonoids (11.17 ± 0.07 nkat/mg for kaempferol, 5.90 ± 0.07 nkat/mg for quercetin, 4.48 ± 0.47 nkat/mg for naringenin and 7.40 ± 0.25 nkat/mg for apigenin (Fig. 3)), which was consistent with our previous observations (Noguchi et al. 2007). GmUGT1 was also capable of acting on luteolin (a flavone; specific activity, 1.67 ± 0.02 nkat/mg), (S)-equol (a 4',7-isoflavandiol; 4.86 ± 0.10 nkat/mg) and (R)-equol (5.53 ± 0.18 nkat/mg). The reaction of GmUGT1 with genistein and daidzein yielded single-transfer products, each of which was co-eluted with genistin and daidzin, respectively, during analytical reversed-phase HPLC. The product peaks of GmUGT1-catalyzed glucosyl transfer to kaempferol and apigenin were identified as kaempferol 4'-O- β -D-glucoside and apigenin 4'-O- β -D-glucoside, respectively, on the basis of their co-chromatography with authentic samples in HPLC (Supplementary Fig. S4) and published spectral properties (Ko et al. 2006).

GmUGT7, another subgroup-B member with the highest sequence similarity to GmUGT1 (Table 1), showed activity toward isoflavones (e.g. 2.64 ± 0.28 nkat/mg for genistein) and toward flavonols and flavones that were comparable. The regiospecificities of glucosyl transfer by GmUGT7 to flavonols and flavones were indistinguishable from those of GmUGT1 (Supplementary Fig. S4). GmUGT8, another subgroup-B member, was catalytically inactive toward all of the flavonoid substrates examined in this study (Fig. 3).

Overall, the members of subgroup A (GmUGT3, GmUGT4, and GmUGT9) were highly specific for isoflavones while two of the subgroup-B members (GmUGT1 and GmUGT7) displayed relatively broad substrate specificities toward flavonoids. The kinetic parameters of these five GmUGTs were determined, and are shown in Table 2.

We examined the glycosyl-donor specificities of GmUGT3 and GmUGT4 and compared them with those previously reported for GmUGT1 (Noguchi et al. 2007). The results showed that UDP-galactose and UDP-glucuronic acid were essentially inert as substrates for GmUGT3 and GmUGT4. This was in contrast to that of GmUGT1, which could act on UDP-galactose (relative activity, 12% of the activity for UDP-glucose).

Transcription analyses

Quantitative real-time RT-PCR with gene-specific primers was performed to compare transcription levels of the flavonoid-active GmUGT homologs (GmUGT1, GmUGT3, GmUGT4, GmUGT7, and GmUGT9) in four different tissues (cotyledons, hypocotyls, taproots, and lateral roots) of one-week-old soybean seedlings (Fig. 4A). The transcription levels of GmUGT1

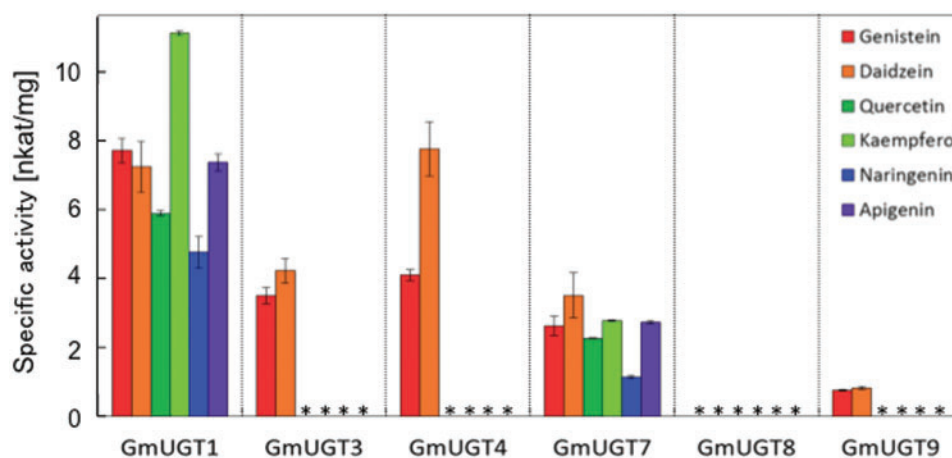


Fig. 3 Substrate specificities of recombinant GmUGTs. Specific glucosyl transfer activities of UmUGTs at pH 8.5 and 30°C for genistein (red bars), daidzein (orange bars), quercetin (green bars), kaempferol (light green bars), naringenin (blue bars), apigenin (purple bars) presented as the average of three independent determinations with \pm SDs. Asterisks, activity not detected.

Table 2 Kinetic parameters^a

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
GmUGT1			
Genistein	4.34 ± 0.60	17.0 ± 2.3	2.55×10^5
Daidzein	5.75 ± 0.47	18.9 ± 2.4	3.04×10^5
UDP-Glc ^b	8.79 ± 0.68	53.7 ± 9.9	1.64×10^5
Kaempferol	1.95 ± 1.01	3.81 ± 0.63	5.11×10^5
GmUGT3			
Genistein	1.75 ± 0.39	12.9 ± 1.2	1.36×10^5
Daidzein	0.97 ± 0.25	10.3 ± 2.4	9.48×10^4
UDP-Glc ^b	1.72 ± 0.50	163.7 ± 60.2	1.05×10^4
GmUGT4			
Genistein	6.04 ± 0.91	26.7 ± 4.8	2.26×10^5
Daidzein	5.89 ± 0.65	20.3 ± 4.2	2.91×10^5
UDP-Glc ^b	7.65 ± 0.92	86.9 ± 21.7	8.80×10^4
GmUGT7			
Genistein	0.39 ± 0.18	2.37 ± 0.92	1.67×10^5
Daidzein	1.22 ± 0.18	9.85 ± 3.50	1.24×10^5
UDP-Glc ^b	1.41 ± 0.20	56.4 ± 13.7	0.25×10^5
GmUGT9			
Genistein	0.13 ± 0.11	0.31 ± 0.12	4.09×10^5
Daidzein	0.10 ± 0.07	0.42 ± 0.16	2.44×10^5
UDP-Glc ^b	0.11 ± 0.07	49.0 ± 17.9	0.02×10^5

^aKinetic parameters were determined at pH 8.5 and 30°C with UDP-glucose (200 μM), as described in the Materials and Methods section.

^b50 μM daidzein was used as the sugar acceptor.

and *GmUGT7* (subgroup-B *GmUGTs*) were higher than those of other homologues in the aerial parts (cotyledons and hypocotyls) as well as in the taproots, whereas in the lateral roots the transcription levels of *GmUGT4* (a subgroup-A *GmUGT*) were significantly higher than those of the other *GmUGTs*. The transcription levels of other subgroup-A *GmUGTs* (i.e. *GmUGT3* and *GmUGT9*) were considerably lower than the other *GmUGTs* in all tissues examined.

Pods and seeds were collected from 12-week-old soybean plants and were analyzed for the transcription levels of *GmUGTs* (Fig. 4B). *GmUGT4* and *GmUGT7* were abundantly expressed in the seeds while *GmUGT1* was the form that was most abundantly expressed in the pods.

It must be mentioned that, in the seedlings, the relative transcription levels of *GmUGT1*, *GmUGT3*, and *GmUGT7* in the roots were, generally, 10-fold lower than those in the aerial parts (Supplementary Fig. 5). However, the levels of the *GmUGT4* transcripts were the highest in the roots, followed by the taproots, the cotyledon, and the hypocotyls. *GmUGT9* showed similar levels of transcription throughout the organs examined. The levels of the most abundant *GmUGT* transcripts in the cotyledons (i.e. *GmUGT1* and *GmUGT7*) were much higher than those in the lateral roots (i.e. *GmUGT4*). The expression levels of these *GmUGT* proteins in the respective organs remained to be examined.

Discussion

Thus far, different physiological roles of isoflavones have been described in different organs and tissues of soybean plants where they occur. For example, in the roots isoflavones are proposed to play pivotal roles in the root nodulation for nitrogen fixation. In soybean leaves, isoflavones and their derivatives serve as antimicrobial agents. Moreover, in soybean seeds, a large amount of isoflavone conjugates accumulates and serves as the most important source of phytoestrogens, which are of nutritional significance in humans. The results obtained in the present study show that two distinct UGT88E subgroups of *GmUGTs* (subgroup A represented by *GmUGT4* and subgroup B represented by *GmUGT1* and *GmUGT7*) could be involved in isoflavone conjugation in an organ/tissue-dependent manner. Although the primary structures of these two enzyme subgroups are similar (e.g. 63% identity between *GmUGT4* and *GmUGT1*), the glucosyl acceptor specificities of these two groups are clearly different—*GmUGTs*

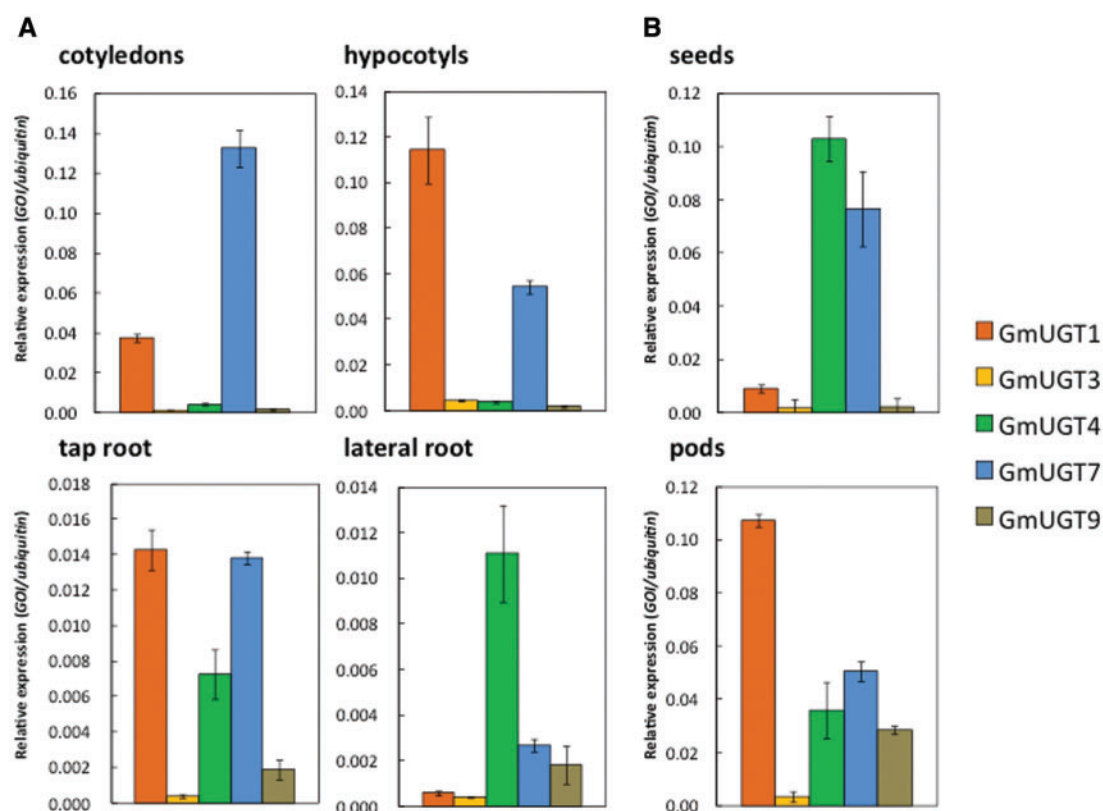


Fig. 4 Relative transcription levels of genes coding for flavonoid-active *GmUGTs* (*GmUGT1*, *GmUGT3*, *GmUGT4*, *GmUGT7* and *GmUGT9*). (A) Transcription levels in the cotyledons, hypocotyls, lateral roots, and taproots of 7-day-old soybean seedlings grown on vermiculite containing a nitrogen-free nutrient solution. (B) Transcription levels of *GmUGTs* in the seeds and pods of 12-week-old soybean plants grown in pots. Transcription levels are expressed as relative values to that of soybean ubiquitin gene (*GmUBQ*). The results are presented as the average of three independent determinations with \pm SDs.

belonging to subgroup A are highly specific for isoflavones while those belonging to subgroup B have broad specificity and efficiently act on other flavonoids, such as flavonols and flavones, besides isoflavones. To find any evolutionary significance in the observed differentiation of *GmUGTs* into these subgroups, molecular evolutionary analyses of *GmUGTs* were performed (Supplementary Fig. S6 and Supplementary Table S3). The d_N/d_S ratios (ω ; the ratios of non-synonymous to synonymous substitutions rates) of these subgroups were estimated by maximum likelihood analysis using PAML 4.8 (Yang 2007) on the basis of the branch model (Yang 1998). The results showed that the d_N/d_S ratios in subgroup B ($\omega = 0.43$) was significantly greater ($P = 0.032$) than the ratios in subgroup A ($\omega = 0.29$) (Supplementary Fig. S6), implying that differentiation into subgroup B (i.e. the gain of broader sugar-acceptor specificity) might have arisen either from positive Darwinian selection or from its higher degree of relaxation of functional constraints. Several candidates for the sites of amino acid substitution that were related to the possible selection could be predicted on the basis of the site model (Nielsen and Yang, 1998) (Supplementary Table S3), although none of these sites displayed a strong statistical significance ($\text{Prob}(d_N/d_S) < 0.99$). Most of these candidates for the sites of amino acid substitution occur in the N-terminal domain, and the sugar acceptor specificity of UGT is thought to be governed by the N-terminal

domain in the entire UGT structure (Bowles et al. 2005). Thus, one or some of these sites might critically determine the sugar acceptor specificity of *GmUGTs*, and this issue will be addressed in our future studies.

It must also be mentioned that the isoflavone-specific UGTs (*PIUGT1* and *PIUGT13*) of *Pueraria lobata*, a leguminous plant, were recently identified and biochemically characterized (Li et al. 2014). These UGTs are members of UGT88 family (UGT88E12 and UGT88H1, respectively; Fig. 2) and are most abundantly expressed in the roots. These *PIUGTs* were highly specific for isoflavones, although they also displayed a weak glucosyl transfer activity toward other flavonoids such as flavanones. Phylogenetically, *PIUGT1* was related to subgroup A of *GmUGTs* (Fig. 2) while *PIUGT13* was distant from subgroups A and B.

Roles of *GmUGT4* in soybean plants

In soybean seedlings, *GmUGT4*, a subgroup-A member, was highly and exclusively expressed in lateral roots (Fig. 4), where the well-known *GmUGT1/GmIF7GT* was expressed at only a very low level, which was consistent with previous observations (Livingstone et al. 2011). An appreciable level of *GmUGT4* expression was also found in taproots, although the expression levels of *GmUGT1* and *GmUGT7* were higher than that of *GmUGT4*. *GmUGT4* was highly specific for isoflavones

and showed a very high value for k_{cat}/K_m ($2.91 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$) for daidzein, the conjugates of which occur predominantly in the lateral roots (Yoo et al. 2013). These observations strongly suggest that GmUGT4, but not GmUGT1, plays an exclusive role in the conjugation of isoflavones in the lateral roots of the soybean plant. Although other subgroup-A members (GmUGT3 and GmUGT9) were also highly specific for isoflavones, the specific activities of these members were lower than that of GmUGT4, and their transcripts accumulated only at low levels in the roots and other organs. The possibility of stress-induced expressions in these two members (such as those caused by wounding, microbial infection, and N-addition/starvation, etc.) remains to be studied.

Lateral roots play a major role in N_2 fixation in the soybean plant, as well as in other leguminous plants, which established the physiological significance of GmUGT4, as follows. Lateral roots expand the root zone of a plant in horizontal directions, and their total rhizoplane area is greater than that of taproots (e.g. the fresh weights of lateral roots and taproots for the 12-week-old soybean plants used in this study were 6.0 ± 1.9 g/plant and 0.15 ± 0.03 g/plant, respectively (A. Funaki, unpublished results)). Root branching provides an increased chance for interactions with symbiotic microorganisms in different soil environments; hence, compared with taproots, lateral roots can provide more nodulation sites. Thus, it is not surprising that the lateral-root nodules, rather than the taproot nodules, contribute to overall N_2 fixation during pod fill (McDermott and Graham 1989). Lateral-root nodules also are known to play a major role in N_2 fixation among other parts of the root systems of common beans (*Phaseolus vulgaris* L.) (Wolyn et al. 1989). This observation illustrates the importance of the lateral-root nodules in N_2 fixation by leguminous plants and underscores the importance of isoflavones that are required for nodulation and are located in the lateral roots. Thus far, isoflavone conjugates are known as the stored precursors of isoflavones (aglycons) that are involved in rhizobia-mediated nodulation (Graham 1991). Among the organs and tissues of soybean seedlings (cv. Enrei), lateral roots consistently accumulate isoflavone conjugates at the highest levels ($\sim 910 \mu\text{g/g Fw}$), with the conjugates of daidzein accounting for $>90\%$ of total isoflavones, followed by taproots ($\sim 610 \mu\text{g/g Fw}$), cotyledons ($\sim 400 \mu\text{g/g Fw}$), and hypocotyls ($\sim 120 \mu\text{g/g Fw}$) (Yoo et al. 2013). Therefore, isoflavone aglycons that are produced in the cytoplasm are likely to be glucosylated by isoflavone-specific GmUGT (GmUGT4) with subsequent malonylation (Suzuki et al. 2007) in the cells of the lateral roots of soybean seedlings. This process is followed by their accumulation in vacuoles, which allows for nodulation and other isoflavone-mediated biological processes.

GmUGT4 is also abundantly expressed in soybean seeds, which is another rich source of isoflavone conjugates in the soybean plant. The content of isoflavone conjugates in the seeds of cv. Enrei was $2518 \mu\text{g/g FW}$ (daidzin, $384 \mu\text{g/g}$; malonyldaidzin, $569 \mu\text{g/g}$; genistin, $566 \mu\text{g/g}$; malonylgenistin, $999 \mu\text{g/g}$; T. Waki, unpublished results). GmUGT4 demonstrates high glucosyl-acceptor specificity toward isoflavones, and is also likely to be involved in the biosynthesis of isoflavone

conjugates in seeds. Thus, GmUGT4 could be responsible for the 7-O-glucosylation of isoflavones in the isoflavonoid-rich organs and tissues of the soybean plant.

Roles of GmUGT1 and GmUGT7 in soybean plants

GmUGT1 and GmUGT7 are flavonoid-active members of subgroup B and display a broad acceptor specificity toward flavonoids, which makes them capable of acting on flavonols, flavones, and flavanones, in addition to isoflavones. These members are highly expressed in tissues and organs other than the lateral roots (i.e. cotyledons, hypocotyls, and taproots) of soybean seedlings, where the conjugates of isoflavones (mainly daidzein conjugates in taproots and genistein conjugates in the cotyledons and hypocotyls) accumulate (see above). The isoflavone conjugates that occur in the cotyledons of soybean seedlings are considered stored precursors of antimicrobial agents. For example, in the cotyledon tissues of soybean seedlings, daidzin and malonyldaidzin occur at a level higher than the effective level of glyceollin (a soybean phytoalexin) and are rapidly hydrolyzed during incompatible infection by the fungal pathogen *Phytophthora sojae* at the infection site (Graham et al. 1990). Moreover, the level of the conjugates (mainly genistin and malonylgenistin) in cotyledons is strongly correlated with the degree of distal defense potentiation against the fungal pathogen. The conjugates are induced by wounding and elicitor treatment (Park et al. 2002). Thus, it is highly likely that GmUGT1 and GmUGT7 play a key role in the storage of the antimicrobial precursors in the cotyledons and hypocotyls of soybean seedlings. Moreover, judging from the activity results and the results of transcription analysis, GmUGT7, along with GmUGT4 (see above), may also be involved in the biosynthesis of isoflavone conjugates in the seeds (Livingstone et al. 2011).

Some organs and/or tissues of the soybean plant also contain the glycosides of flavonol and flavone. In soybean leaves, a programmed shift of flavonoid biosynthesis is known to occur from the isoflavone pathway to the flavonol pathway 3 days after germination (Graham 1991). Soybean cotyledons are known to contain kaempferol 3-O-glucosides and related glycosides, which are also the major aromatic metabolites of older leaves (Ho et al. 2002). By contrast, soybean pods are known to contain 7-O-glucosides of flavones (apigenin and luteolin) (Boue et al. 2003). In the present study, GmUGT1 and GmUGT7 were abundantly expressed in cotyledons and pods, and the recombinant enzymes displayed a high degree of relative activity toward kaempferol and apigenin, but the transfer products with these flavonoids were not co-eluted with kaempferol 3-O-glucoside and apigenin 7-O-glucoside in our analytical HPLC system (Supplementary Fig. S4). The most likely to co-elute were kaempferol 4'-O-glucoside and apigenin 4'-O-glucoside. Thus, it is unlikely that GmUGT1 and GmUGT7 are involved in the biosynthesis of glycosides from flavonol and flavones in these organs and tissues.

Although we failed to find any catalytic activity with GmUGT8 protein (see the Results section), we observed that GmUGT8 was abundantly expressed in hypocotyls and taproots (Supplementary Fig. S7). Thus, the possibility remains

that it might also be involved in the (iso)flavonoids metabolism and this issue has to be addressed in future studies.

In conclusion, the results obtained in this study illustrate the differential expressions of UGT88-related UGTs in an organ/tissue-dependent manner in soybean plants. In soybean seedlings, the 7-O-glucosylation of isoflavones (mainly daidzein) is exclusively catalyzed by the isoflavone-specific member GmUGT4 in the lateral roots, while the 7-O-glucosylation of isoflavones in other parts is catalyzed by GmUGT1 and GmUGT7.

Materials and Methods

Plant materials and chemicals

Uniform, unblemished, disease-free soybean seeds (cv. Enrei) were surface-sterilized with 70% ethanol for 30 sec followed by 0.04% hypochlorite solution for 8 min. The seeds were sown in sterile vermiculite with a nitrogen-free plant nutrient solution (Akao and Kouchi 1989) in sterile Leonard jar assemblies composed of two 300-ml plant boxes (Ye et al. 2005), and were grown for 3 d at 25°C in the dark. The plants were further cultivated in uncovered Leonard jar assemblies for 5 d at 25°C under long-day conditions (16 h light and 8 h dark) in a plant growth cabinet (Plant Environmental Control System model KCLP-1400 II CT, Nippon Medical & Chemical Instruments, Osaka, Japan), which provided $\sim 200 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ of white light irradiation. The roots of 7–9 day-old seedlings were washed with tap water to remove soils and were frozen at -80°C until used. Soybean plants were also sown in pots and grown to maturity. Pods (approximately 5 cm in length) were harvested in triplicate from individual plants.

Naringenin was purchased from Nacalai Tesque (Kyoto, Japan). Kaempferol and quercetin were obtained from Wako Pure Chemical Industries (Osaka, Japan). Apigenin and luteolin were from the Extrasynthese (Lyon, France) and LKT Laboratories (St. Paul, MN, USA). Genistein, genistin, daidzein and daidzin were products of Fujicco (Kobe, Japan). Optical isomers of equol were kindly provided by Daicel Corp. (Tokyo, Japan). 3-O-Glucosides of kaempferol, 3-O- and 4'-O-glucosides of quercetin, 7-O-glucoside of naringenin, and 7-O-glucoside of apigenin were obtained from Extrasynthese. UDP-glucose, UDP-galactose and UDP-glucuronic acid were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

Cloning of GmUGT homologs

The Phytozome database v9.1 (*G. max*) (http://www.phytozome.net/search.php?show=text&method=Org_Gmax) (Schmutz et al. 2010) was screened *in silico* using standard BLAST algorithms (Altschul et al. 1990) and the GmUGT1 nucleotide sequence. GmUGT1, GmUGT2, GmUGT3, GmUGT4, GmUGT5, and GmUGT6 corresponded to GmIF7GT1, GmIF7GT2, GmIF7GT3, GmIF7GT4, GmIF7GT5 and GmIF7GT6, respectively, which were reported by Livingstone et al. (Livingstone et al. 2011) and GmUGT7, GmUGT8 and GmUGT9 were the UGT genes that were newly cloned and characterized in this study.

Because all of the genomic sequences of our target GmUGT genes (GmUGT1, GmUGT3, GmUGT4, GmUGT7, GmUGT8 and GmUGT9) had no intron, we used the soybean genomic DNA as a template for PCR amplification of these coding regions. Genomic DNA of *G. max* cv. Enrei was prepared from the cotyledon (approximately 0.1 g) of 7-day-old seedlings according to a method established by Pich and Schubert (1993). Amplification of each GmUGT was performed by PCR using the primers shown in **Supplementary Table S1** (A) with the genomic DNA (50 μg) as a template, followed by nested PCR using the primers shown in **Supplementary Table S1** (B). The thermal cycling conditions were 94°C for 2 min followed by 30 cycles of PCR (one cycle consisted of 98°C for 10 sec, 55°C for 30 sec and 68°C for 45 sec). The PCR product, 1.4 kb in length in each case, was gel-purified and cloned into pGEM-T Easy vector (Promega, WI, USA). The nucleotide sequences of the DNA inserts for the resultant recombinant plasmids (pGEM-T Easy-UGT3 through pGEM-T Easy-UGT9) were confirmed by DNA sequencing.

Heterologous expression and purification of the expressed products

The pGEM-T Easy-UGT4 plasmid, e.g. was digested with NdeI and BamHI to obtain the full-length GmUGT4 CDs. The cDNA was ligated into a pCold-I vector (Takara Bio, Shiga, Japan) that had previously been digested with NdeI and BamHI to obtain the plasmid pCold-I-GmUGT4, which encoded an N-terminal in-frame fusion of GmUGT4 with a His₆ tag. The plasmids pCold-I-GmUGT7, pCold-I-GmUGT8, and pCold-I-GmUGT9 were prepared in the same way described above. For construction of the pCold-I-GmUGT3 plasmid, the pGEM-T Easy-UGT3 plasmid was digested with NdeI and SalI, and the resultant cDNA was ligated with the NdeI/SalI-digested pCold-I vector. *E. coli* BL21 cells were transformed with the resultant plasmid.

Heterologous expression of each GmUGT cDNA (GmUGT1, GmUGT3, GmUGT4, GmUGT7, GmUGT8 and GmUGT9) was carried out as follows. After the transformants were pre-cultured at 37°C for 16 h in Luria-Bertani broth containing 50 $\mu\text{g/ml}$ ampicillin, the culture was used to inoculate the same medium (40 ml). After cultivating the cells with shaking (180 r.p.m.) at 37°C until the optical turbidity at 600 nm of the culture reached 0.5, isopropyl 1- β -D-thiogalactoside was added to the medium at a final concentration of 1.0 mM, followed by cultivation with shaking (180 rpm) at 15°C for 24 h. The cells were then harvested by centrifugation at 5,000 $\times g$ and 4°C for 10 min.

All subsequent operations were conducted at 0 – 4°C . The harvested cells were suspended in buffer A (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing 1 mg/ml lysozyme, 20 mM imidazole, and a recommended dilution of a proteinase inhibitor cocktail (Nacalai Tesque). The cell suspension was chilled on ice for 1 h. The cells were then disrupted at 4°C by 5 cycles of ultrasonication, where one cycle corresponded to 20 kHz for 10 sec followed by an interval of 30 sec. The resultant cell debris was removed by centrifugation at 15,000 $\times g$ and 4°C for 10 min, followed by filtration with a 0.45- μm filter (Millipore, Billerica, MA, USA). The supernatant was applied to a 1-ml HisTrap HP column (GE Healthcare Life Sciences, Tokyo, Japan) that had been equilibrated with buffer A containing 20 mM imidazole. The column was washed with 10 ml of buffer A containing 100 mM imidazole, followed by washing with 5 ml of buffer A containing 300 mM imidazole. The enzyme-containing fractions were concentrated using an Amicon Ultra-4 (10 K) ultra-filtration device (Millipore), followed by substitution with 50 mM Tris-HCl buffer, pH 8.5. SDS-PAGE was carried out according to a method established by Laemmli (1970), and the proteins in the gels were visualized using Coomassie Brilliant Blue R250.

Enzyme and protein assays

UDP-glucose:flavonoid glucosyltransferase activity was measured using flavonoid and UDP-glucose as substrates. A standard reaction mixture (200 μl) consisted of 50 μM flavonoid, 200 μM UDP-glucose, 50 mM Tris-HCl, pH 8.5 and an enzyme. The mixture without an enzyme was preincubated at 30°C for 10 min, and the reaction was started by the addition of an enzyme. After incubation at 30°C for 10 min, the reaction was stopped by the addition of 8 μl of 6.5% (v/v) trifluoroacetic acid. The reaction products were analyzed using a Gilson 305 HPLC system on a J'sphere ODS-M80 column (4.6 \times 150 mm, YMC, Kyoto, Japan), where solvent A was 0.1% phosphoric acid in a 1:9 (v/v) mixture of acetonitrile and water and solvent B was 0.1% phosphoric acid in a 9:1 (v/v) mixture of acetonitrile and water. The substrates and products were eluted with a linear gradient of 10% B to 60% B in 10 min at a flow rate of 0.8 ml/min. The chromatograms were obtained with detections at 260 nm (for isoflavones), 290 nm (for flavanones), 340 nm (for flavones), 360 nm (for flavonols) and 280 nm (for equol) using a SPD M-20A UV-visible detector (Shimadzu, Kyoto, Japan). The protein concentration was determined via the method established by Bradford (Bradford 1976) using bovine serum albumin as a standard.

Enzyme kinetics

The initial velocity assays for GmUGT homologs were carried out under steady-state conditions using a standard assay system (see above) with varying concentrations of substrates. Apparent K_m and k_{cat} values of glucosyl donor and acceptor substrates were determined in the presence of a constant concentration of their counter substrate by using a computer program (SigmaPlot) to fit the initial velocity data to a Michaelis-Menten equation for single-substrate enzyme reactions.

Transcription analysis

RNA was isolated from the individual organs of soybean seedlings using reagents RNAiso Plus and Fruit-mate for RNA Purification (TaKaRa Bio) according to the manufacturer's guidelines. Contaminating DNA in the total RNA sample was removed by treatment with DNase I (RNase-free, TaKaRa Bio). RNA was quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Yokohama, Japan). Reverse transcription was performed in 10- μ l reactions using the PrimeScript RT reagent Kit (Perfect Real Time) (TaKaRa Bio) as per the manufacturer's instructions. Real-time quantitative PCR (10 μ l) were then run on the Eco Real Time PCR System (Illumina, Tokyo, Japan) using gene-specific primers ([Supplementary Table S2](#)) and Fast SYBR Green Master Mix (Life Technologies, Tokyo Japan) following the manufacturer's instructions. Cycling conditions included an initial hot start at 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec and at 60°C for 30 sec. A temperature of 64°C was used for the annealing and extension primer sets for *GmUGT7*. Each PCR reaction was followed by a melting curve program to check that only single products were amplified, starting with denaturation at 95°C for 1 sec before cooling to 65°C for 15 sec then increasing with continuous fluorescence measurement until 95°C was reached. Negative controls consisted of water in place of cDNA and were run with all reactions. Data were analyzed using Eco Software (ver. 3.0, Illumina). A standard curve was generated using a serial dilution of plasmid containing each gene, and the resultant efficiencies were used to calculate the expression of soybean ubiquitin (*GmUBQ*, DDBJ/ENA/GenBank accession number X13251).

Supplementary data

Supplementary data associated with this article can be found in the online version, at PCP Online.

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