BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Regioselective synthesis of plant (iso)flavone glycosides in *Escherichia coli*

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Abstract The flavonoids genistein, biochanin A, luteolin, quercetin, and kaempferol are plant natural products with potentially useful pharmacological and nutraceutical activities. These natural products usually exist in plants as glycosides, and their glycosylation has a remarkable influence on their pharmacokinetic properties. The glycosyltransferases UGT71G1 and UGT73C8 from *Medicago truncatula* are excellent reagents for the regioselective glycosylation of (iso)flavonoids in *Escherichia coli* grown in Terrific broth. Ten to 20 mg/L of either genistein or biochanin A 7-*O*-glucoside was produced after feeding genistein or biochanin A to *E. coli* expressing UGT71G1, and similar levels of luteolin 4'-*O*- and 7-*O*-glucosides were produced after feeding luteolin to cultures expressing UGT73C8. For the production of kaempferol 3-*O*-glucoside or quercetin 3-*O*-glucoside, the Phe148Val or Tyr202Ala mutants of UGT71G1 were employed. Ten to 16 mg/L of either kaempferol 3-*O*- or quercetin 3-*O*-glucosides were produced on feeding kaempferol or quercetin to *E. coli* expressing these enzymes. More than 90% of the glucoside products were released to the medium, facilitating their isolation.
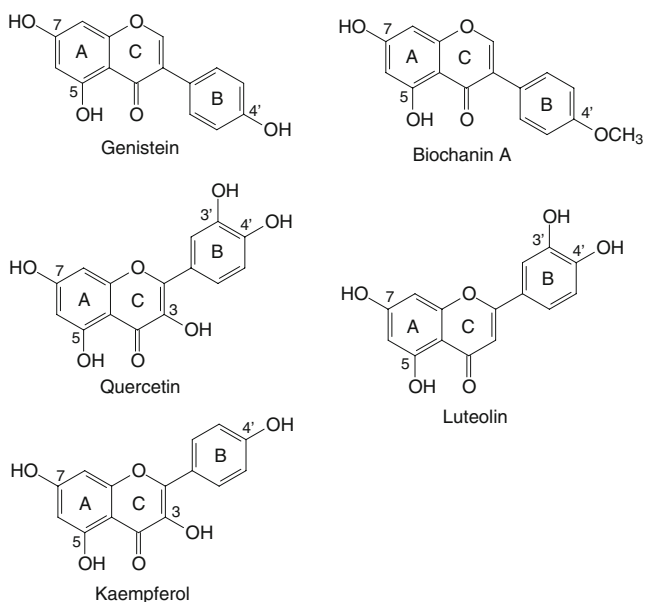
Introduction

Polyphenolic flavonoids are common constituents of vegetables, fruits, and herbal medicines. Their health-promoting effects have long been recognized, and flavonoids such as genistein, biochanin A, quercetin, kaempferol, and luteolin have been exploited for their medicinal and nutritional activities (Arai et al. 2000; Erdman et al. 2007; Mink et al. 2007). The isoflavone genistein, biochanin A, and the flavonols quercetin and kaempferol (Fig. 1) have been shown to reduce the incidence of cancer (Caltagirone et al. 2000; Kao et al. 1998; Kellis and Vickerry 1984; Shenouda et al. 2004; Way et al. 2004) and cardiovascular disease (Arai et al. 2000; Cogolludo et al. 2007; Hertog et al. 1993; Mink et al. 2007) and also to exhibit anti-inflammatory activities (Gabor 1979; Kumazawa et al. 2006; Lewis 1989). Many studies have been aimed at developing new production platforms for such plant natural products through metabolic engineering in plants or microorganisms (Deavours and Dixon 2005; Leonard et al. 2005, 2006; Lim et al. 2004; Liu et al. 2002; Willits et al. 2004; Yu et al. 2000, 2003).

Most phenolic secondary metabolites exist in plants as glycosides, often with more than one hydroxyl group glycosylated. Metabolic engineering by expression of isoflavone synthase in *Arabidopsis*, tobacco, soybean, and alfalfa leads to accumulation of genistein glycosides rather than the aglycone (Deavours and Dixon 2005; Liu et al. 2002; Yu et al. 2000, 2003). Glycosylation of the aglycones increases their solubility and stability (Hollam et al. 1995, 1996, 1999; Smith et al. 2000) and also increases their bioavailability in mammals (Crespy et al. 2001; Graefe et al. 2001; Hollman and Katan 1998, 1999). Furthermore, the position of glycosylation, for example 4'-*O*-, 3-*O*-, or 3-, 4'-di-*O*-glucosides in the case of quercetin, significantly impacts hydrolysis and bioavailability (Cermak et al. 2004; Day et al. 2003; Gee et al. 2000). For example, quercetin 3-*O*- and 4'-*O*-glucosides, but not the 3-,4'-*O*-diglucoside, were transported through the rat small intestine, and during passage across the epithelium the monoglucoside was

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Fig. 1 Chemical structures of the isoflavones genistein and biochanin A, the flavone luteolin, and the flavonols quercetin and kaempferol

rapidly deglycosylated and then glucuronidated into quercetin 3-O- and 7-O-glucuronide (Gee et al. 2000).

Availability of a range of (iso)flavonoids with different regiospecific glycosyl substituents will facilitate research on their biological utilization. However, regiospecific chemical synthesis of polyphenolic glycosides is far from trivial (Bouktaib et al. 2002; Li et al. 2002). We have previously characterized the glycosyltransferases UGT71G1 and UGT73C8 from the model legume *Medicago truncatula* (Achnine et al. 2005; Modolo et al. 2007; Shao et al. 2005). UGT71G1, a member of group E family 1 glycosyltransferases, regioselectively glycosylates the 7-O position of genistein and biochanin A but produces five different monoglucosides from quercetin, with the 3'-O-glucoside predominating (Achnine et al. 2005; He et al. 2006). The structure of UGT71G1 has been determined and belongs to the GT-B fold with Rossmann-like domains (Shao et al. 2005). Two structure-directed mutants of the enzyme, Phe148Val and Tyr202Ala, predominantly glycosylated the 3-O position of quercetin in vitro (He et al. 2006). UGT73C8, closely related to UGT72C1 (72% identity) in group D of family 1 glycosyltransferases, showed enzyme activity preference for isoflavonoids but also exhibited high activity with the flavone luteolin (Modolo et al. 2007). However, the regioselectivity and effectiveness of these enzymes as biocatalysts for the synthesis of (iso) flavonoid glycosides in vivo remains to be evaluated. Here, we report the production of milligram quantities of the 7-O-glucosides of genistein and biochanin A, the 4'-O- and 7-O-glucosides of luteolin, and the 3-O-glucosides of quercetin and kaempferol in *Escherichia coli* cultures

expressing UGT71G1, UGT73C8, and mutants of UGT71G1, respectively.

Materials and methods

Bacterial growth, induction, precursor feeding, and product extraction

General chemicals and quercetin 3-O-glucoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other (iso) flavones and their glycosides were purchased from Indofine Chemicals (Hillsborough, NJ, USA). Kaempferol 4'-O-glucoside was purchased from Extra-synthese (Genay, France). The protein expression vector pET28a and *E. coli* strain BL21(DE3) were from Novagen (Madison, WI, USA).

The glycosyltransferases UGT71G1, UGT73C8, and UGT71G1 mutants Phe148Val and Tyr202Ala were cloned into pET 28a as described previously (He et al. 2006). *E. coli* BL21 (DE3) strains carrying these constructs were grown in Terrific broth (TB) or Luria-Bertani (LB) medium at 37°C until OD_{600} reached 0.7. Isopropyl-1-thio-β-D-galactopyranoside was then added to the cultures to a final concentration of 0.5 mM, and the cultures were incubated at 20°C for 5 h. Substrate (genistein, biochanin A, luteolin, quercetin, or kaempferol) dissolved in dimethyl sulfoxide (DMSO) was then added to the cultures to a final concentration of 50 or 100 μM. *E. coli* cultures harboring empty vector pET 28a vector were used as a control. Cells were harvested at different time points after addition of substrates. The medium and bacterial cells were separated by centrifugation for 30 min at 4,000×g at 4°C. The medium was acidified with 6 N HCl to pH 1, extracted twice with ethyl acetate, and dried under nitrogen gas. Residues were resuspended in methanol for high-performance liquid chromatography (HPLC) analysis. The cell pellet was resuspended in MeOH–HCl (v/v, 10:1) and extracted for 1 h in an ultrasonic bath as described previously (Willits et al. 2004). Insoluble cell debris was removed by centrifugation and the extract was dried under nitrogen gas. Residues were resuspended in methanol for HPLC analysis. For large-scale cultures, 500 mL of bacterial culture were grown in a 2-L flask in TB medium. Bacterial induction was as described above. Twenty-four hours after the addition of substrate (100-μM final concentration), cells were harvested and products were extracted and analyzed as described above.

Soluble protein purification

UGT71G1, UGT71G1 mutants F148V and Y202A, and UGT73C8 soluble proteins were purified from induced cell

cultures using the MagneHis protein purification system according to the manufacturer's instructions (Promega, Madison, WI, USA). Protein concentration was determined with the Bio-Rad protein dye-binding assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) using bovine serum albumin as standard.

HPLC analysis of glycosylated products

Reverse-phase HPLC analysis was carried out on a Hewlett Packard 1100 system using a 5-μm C18 column (250× 4.6 mm, Waters spheroisorb 5 μm ODS2) with the following gradient: A = 1% phosphoric acid, B = acetonitrile, 0–5 min, 5%B; 5–10 min, 10% B; 10–25 min, 17% B; 25–30 min, 23% B, 30–65 min, 50% B; 65–79 min, 100% B; 79–80 min, 5% B. The eluate was monitored at 254 nm. The glycosylated products were identified according to their retention times, UV spectra, and comparison to authentic standards (Lim et al. 2004).

Results

### Production of genistein and biochanin A 7-O-glucoside in E. coli

UGT71G1 is a multifunctional triterpene–flavonoid glycosyltransferase with specific activity for the triterpene medicagenic acid, the isoflavones genistein and biochanin A, and the flavonol quercetin. Recombinant *M. truncatula* UGT71G1 can transfer glucose from uridine diphosphate (UDP)-glucose to genistein or biochanin A to produce genistein or biochanin A 7-O-glucoside in vitro (Achnine et al. 2005; He et al. 2006; Shao et al. 2005). To investigate the production of genistein or biochanin A 7-O-glucosides in *E. coli* in vivo, we incubated induced cell cultures of *E.*

coli BL21(DE3) carrying the UGT71G1–pET 28a construct with different concentrations of genistein or biochanin A for different times. The medium and cell pellet were extracted with ethyl acetate, and products were analyzed by HPLC. The expected 7-O-glucosides were produced under all conditions used and could be detected in the culture medium after 5 h (Fig. 2, Table 1). More than 70% of genistein substrate was converted to the 7-O-glucoside by the *E. coli* cultures after 24-h incubation. The product yield was about 20-mg/L TB culture medium after 24-h incubation with 100 μM (27.02 mg/L) genistein. In comparison to LB culture medium, a 3.5-fold higher 7-O-glucoside yield was achieved in TB culture medium, with twice as much glucoside produced with 100 μM as compared to 50 μM genistein after 24-h incubation (Fig. 2, Table 1). A similar glucoside production pattern was observed when the culture was incubated with biochanin A. Thirteen milligram per liter biochanin A 7-O-glucoside was produced after 24-h incubation with 100 μM (28.42 mg/L) biochanin A substrate in TB medium. About threefold higher levels of 7-O-glucoside were produced in TB medium compared to LB medium (Fig. S1, Table 1). No genistein or biochanin A 7-O-glucoside was detected in the medium from *E. coli* harboring the vector control (Figs. S2 and S3).

To evaluate production efficiency on a larger scale, we set up 500-mL TB medium cultures, expressing the corresponding glycosyltransferases or the mutants described above, in 2-L flasks. The substrates were incubated individually for 24 h in the cell cultures and the glucoside products were analyzed by HPLC. The glucoside product yield was about 10–16-mg/L culture medium with conversion rates from 30% to 60% (Table 2). The efficiency was about 80% to that of the small-batch scale. In both cases, more than 90% of the glycosylated products were released to the medium.

To evaluate the effects of the (iso)flavonoid substrates on the growth of *E. coli*, we monitored bacterial growth at

Fig. 2 Production of genistein 7-O-glucoside using UGT71G1 as a biocatalyst in *E. coli*. Time course showing levels of genistein 7-O-glucoside in culture medium of engineered *E. coli* expressing UGT71G1 fed with genistein as substrate (*squares* = 7-O-glucoside level in TB medium, *diamonds* = 7-O-glucoside level in LB medium). *Insert* showed the levels of aglycone consumed during the time course (*triangles* = aglycone level in TB medium, *circles* = aglycone level in LB medium). Fifty micromolar (13.51-mg/L final concentration) genistein added to the bacterial culture
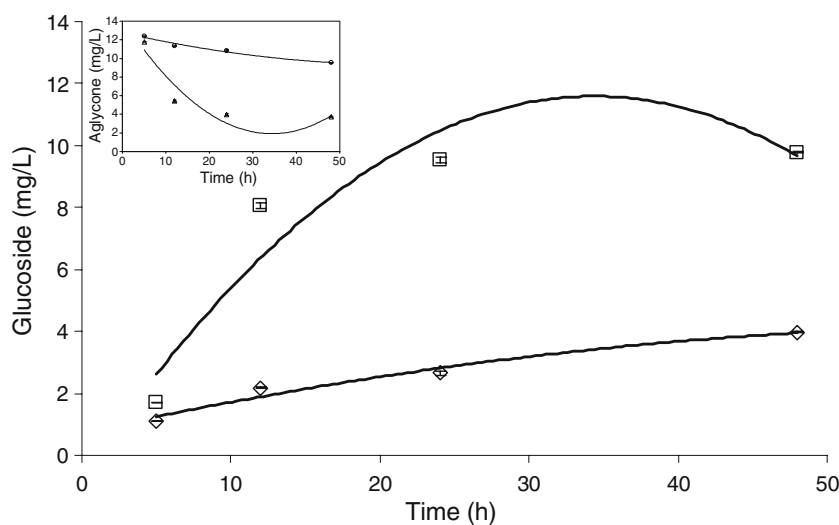
Table 1 (Iso)flavone glucoside synthesis in *E. coli* in LB and TB culture media (100 µM substrate)

Construct	Glucoside	LB medium (mg/L)				TB medium (mg/L)			
		5 h	12 h	24	48 h	5 h	12 h	24 h	48 h
UGT71G1 wild type	Genistein 7-*O*-tglucoside	2.05±0.02	4.39±0.14	5.57±0.07	5.58±0.13	3.95±0.03	15.45±0.28	20.06±0.16	17.90±0.61
UGT71G1 wild type	Biochanin A 7-*O*-tglucoside	2.08±0.03	3.77±0.16	4.05±0.33	4.16±0.10	2.19±0.09	9.50±0.24	13.02±0.57	13.18±0.14
UGT71G1 mutant F148V	Quercetin 3-*O*-tglucoside	2.94±0.06	6.55±0.64	8.20±0.32	5.95±0.55	2.00±0.08	5.93±0.22	11.70±0.03	16.75±0.65
UGT71G1 mutant Y202A	Quercetin 3-*O*-tglucoside	4.15±0.37	9.84±0.21	10.09±0.22	7.86±0.87	1.81±0.08	6.84±0.12	12.37±0.06	19.88±1.26
UGT71G1 mutant F148V	Kaempferol 3-*O*-tglucoside	1.05±0.07	3.56±0.04	3.92±0.07	5.06±0.02	1.71±0.04	10.59±0.35	15.81±0.40	14.83±0.19
UGT71G1 mutant Y202A	Kaempferol 3-*O*-tglucoside	1.18±0.03	3.82±0.07	7.14±0.09	7.81±0.16	2.10±0.08	12.40±0.14	16.46±0.46	18.59±0.41
UGT73C8 wild type	Luteolin 4'-*O*-tglucoside	1.56±0.11	8.45±0.38	9.82±0.24	13.68±0.19	2.78±0.04	11.78±0.06	14.01±0.44	15.49±0.34
UGT73C8 wild type	Luteolin 7-*O*-tglucoside	1.00±0.02	4.76±0.04	4.96±0.03	7.95±0.07	1.26±0.01	7.02±0.05	8.04±0.15	8.91±0.21

each time point during the culture process. There was no effect of substrates on bacterial growth. However, TB medium supported higher bacterial growth than LB medium. An example of these results with genistein is shown in Fig. 3. Soluble cellular recombinant UGT protein was also purified from the induced cell cultures grown with or without substrate to evaluate the effect of substrate on protein levels. Results showed that substrate addition did not have any significant effect on protein expression level, with 0.4–0.59 and 0.13–0.21 µg/µl soluble recombinant protein being obtained in TB and LB medium, respectively, irrespective of substrate addition (Fig. S4, Table 3). UGT71G1 mutant F148V produced similar amounts of soluble protein in both media (data not shown).

Production of luteolin 4'-*O*- and 7-*O*-glucosides

Numerous studies have shown that luteolin [5,7,3'4'-tetrahydroxy flavone (Fig. 1)] has potent anti-inflammatory properties both in vitro and in vivo (Backhouse et al. 2007; Chen et al. 2007; Karrasch et al. 2007; Kim and Jobin 2005). Luteolin inhibits the expression and production of inflammatory associated genes and mediators such as cyclooxygenase-2, tumor necrosis factor-alpha, and interleukin-6, suggesting its possible therapeutic application for treating inflammatory disorder (Chen et al. 2007). UGT73C8 can glycosylate luteolin to produce the corresponding 4'-*O*- and 7-*O*-monoglucosides in vitro (Modolo et al. 2007). When luteolin (100 µM, equal to 28.64 mg/L) was incubated for 24 h with *E. coli* expressing UGT73C8 and grown in TB medium, about 14 mg/L 4'-*O*-glucoside and 8 mg/L 7-*O*-glucoside, respectively, was recovered from the medium (Fig. S5, Table 1). Similar product yield was obtained when the culture was incubated for 48 h. The total glucoside conversion rate was about 75% in small-batch culture and 60% in the 500-mL culture (Table 2). About 1.5-fold higher product yield was obtained in TB medium compared to LB medium. No luteolin

Table 2 (Iso)flavone glucoside synthesis in *E. coli* in 500-mL TB culture medium

Construct	Glucoside	(mg/L)	Conversion rate(%)
UGT71G1 wild type	Genistein 7-*O*-glucoside	16.39±0.30	60.7
UGT71G1 wild type	Biochanin A 7-*O*-glucoside	11.70±0.35	41.2
UGT71G1 mutant F148V	Quercetin 3-*O*-glucoside	10.08±0.10	29.8
UGT71G1 mutant Y202A	Quercetin 3-*O*-glucoside	11.54±0.34	34.2
UGT71G1 mutant F148V	Kaempferol 3-*O*-glucoside	12.65±0.57	44.2
UGT71G1 mutant Y202A	Kaempferol 3-*O*-glucoside	13.56±0.37	47.4
UGT73C8 wild type	Luteolin 4'-*O*-glucoside	10.86±0.08	37.9
	Luteolin 7-*O*-glucoside	6.52±0.13	22.8

The substrates (100-µM final concentration) were incubated in the cell culture for 24 h.

Fig. 3 Growth of *E. coli* BL21 (DE3) carrying plasmid pET28a-UGT71G1 in TB medium with 50 μM genistein (*filled diamonds*), 100 μM genistein (*filled squares*), and no substrate added (*filled triangles*), and in LB medium with 50 μM genistein (*cross marks*), 100 μM genistein (*asterisks*) and no substrate added (*filled circles*)
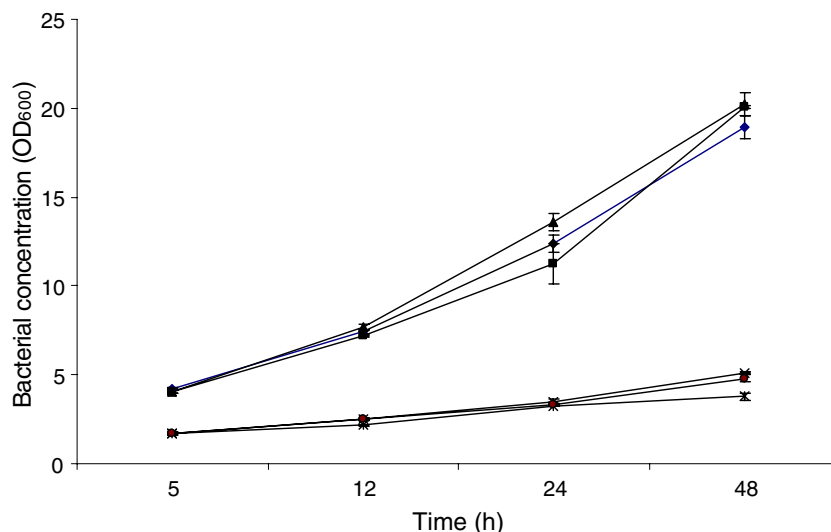
glucoside was observed in the vector control culture (Fig. S6).

Production of quercetin 3-*O*-glucoside in *E. coli*

Quercetin has a hydroxyl group at the 3-position of its C-ring (Fig. 1), and this may be important for its anticarcinogenic activity (Ichimatsu et al. 2007; Shen et al. 2003). Glycosylation of quercetin has been reported to increase its absorption in humans (Hollman and Katan 1998, 1999; Hollman et al. 1999). Recombinant wild-type UGT71G1 produces all five potential monoglucosides from quercetin and UDP-glucose in vitro, with the B-ring 3'-*O*-glucoside predominating (He et al. 2006; Shao et al. 2005). However, the Phe148Val and Tyr202Ala mutants of UGT71G1 produced quercetin 3-*O*-glucoside as the major product in vitro (>95% of the total products; He et al. 2006). In the present study, feeding of quercetin to *E. coli* expressing Phe148Val or Tyr202Ala mutants in TB medium led to production of quercetin 3-*O*-glucoside as the only product

Table 3 Levels of soluble recombinant UGT proteins in *E. coli* cells grown with and without added substrates

	μg/μL (TB)	μg/μL (LB)
UGT71G1	0.48	0.15
UGT71G1 + genistein	0.57	0.19
UGT71G1 + biochanin A	0.49	0.15
UGT71G1 + kaempferol	0.45	0.13
Mutant Y202A	0.43	0.17
Mutant Y202A + quercetin	0.40	0.14
Mutant Y202A + kaempferol	0.46	0.19
UGT73C8	0.59	0.21
UGT73C8 + luteolin	0.56	0.20

The final concentration of substrate is 100 μM. The soluble protein was purified from TB and LB medium, respectively.

detected. About 8- and 17-mg/L culture medium of quercetin 3-*O*-glucoside was produced from the cultures expressing the Phe148Val mutant when incubated with 50 μM (16.91 mg/L) and 100 μM (33.82 mg/L) quercetin, respectively, for 48 h, while production was 10- and 20-mg/L culture with the Tyr202Ala mutant, respectively (Fig. S7, Table 1). The conversion rate from aglycone to glucoside was about 50% after 48 h. In comparison to LB medium, TB medium supported two to threefold higher glycoside production.

Production of kaempferol 4'-*O*- and 3-*O*-glucosides

Kaempferol is a common antioxidant in vegetables and fruits. Compared to quercetin, it contains only a single hydroxyl group (at 4') on its B-ring (Fig. 1). Kaempferol was converted to a mixture of two monoglucosides, the 4'-*O*-glucoside and 3-*O*-glucoside, when fed to *E. coli* expressing wild-type UGT71G1, with yields of about 20 mg 4'-*O*-glucoside and 3 mg 3-*O*-glucoside per liter of TB culture medium, respectively. However, a single product, the 3-*O*-glucoside, was formed when kaempferol was fed to cell cultures expressing the Phe148Val or Tyr202Ala mutants, with yields of about 16-mg/L TB culture medium (Fig. S8, Table 1).

Discussion

The isoflavone genistein and biochanin A, the flavone luteolin, and the flavonols quercetin and kaempferol have beneficial health effects for humans with regards to the prevention of cancer and cardiovascular diseases and anti-inflammatory properties. Glycosylation of these compounds may significantly impact their solubility, absorption, and biological activity. This study was designed to evaluate the

regioselectivity and effectiveness of the plant glycosyl-transferases UGT73C8, UGT71G1, and mutants thereof for production of (iso)flavonoid glycosides in vivo and thus to explore the utilization of the enzymes as regioselective glycosylation biocatalysts. Genistein, biochanin A, luteolin, quercetin, and kaempferol were useful substrates for assessing regioselectivity because of the presence of multiple hydroxyl groups on these compounds. Further-more, there is a need for production of various glycosides of these compounds to evaluate in tests with various animal disease models.

Our results showed that *E. coli* expressing UGT73C8, UGT71G1, or mutants of UGT71G1 collectively can regioselectively glycosylate genistein, biochanin A, luteolin, quercetin, and kaempferol to produce a specific set of glycosides in vivo. Regioselective glycosylation of these compounds by chemical approaches requires sequential blocking and deblocking of the hydroxyl groups. For example, chemical synthesis of quercetin 3-*O*-glucoside first required the selective protection of the catechol hydroxyl groups then glycosylation of the 3-hydroxyl group followed by deblocking of the catechol hydroxyl groups. The procedure gave a 54% yield with contamination from product glycosylated on the 7-hydroxyl group (Bouktaib et al. 2002). On the other hand, direct isolation of significant amounts of specific flavonoid glycosides from plant sources is time-consuming since plants contain wide and variable spectra of glycosides with different types of sugar attachment (Harborne and Baxter 1999).

The enzyme system we describe here allows us to overcome many of the problems associated with efficient production of specific glucosides. Importantly, the glyco-sides are released to the medium, facilitating their isolation. Given that commercially available flavonoid glycosides are currently at least ten times more expensive than their corresponding aglycones expressing regioselective glyco-syltransferases in *E. coli* in vivo is an economically feasible approach to the production of glycosides. The conversion rates in small-batch scale were from 50% to 80%. However, in larger-scale production (500-mL cultures,) the efficiency was reduced to 80% of the small-batch scale. Clearly, further optimization of the conditions will be needed for industry-scale production to increase the product yield.

Nucleotide-activated sugar is the essential component in small molecule glycosylation. One of the major problems for large-scale application of glycosylation is the provision of UDP-glucose to the in vitro system. Although different approaches including chemical methods (Kretzschmar and Stahl 1998), enzymatic synthesis (Bulter and Elling 1999), and regeneration using UDP-glucose pyrophosphorylase and pyrophosphatase for synthesis of nucleoside diphos-phate sugars (Heidlas et al. 1992; Ichikawa et al. 1992; Wong et al. 1992) have been reported, these approaches are

either laborious, difficult, or require sugar phosphates, phosphoenolpyruvate, and nucleotide 5'-triphosphate and are therefore of high cost. Since UDP-glucose is a natural intermediate in cell wall synthesis in bacterial cells, using glycosyltransferases engineered in bacteria to synthesize small molecule glycosides should be an efficient approach to overcome the difficulty associated with the preparation of nucleotide sugars.

Similar studies on production of flavonoid glycosides in *E. coli* have recently been reported (Lim et al. 2004; Willits et al. 2004). *E. coli* expressing UGT73B2 from *Arabidopsis*, with 24% sequence identity to UGT71G1, produced primarily quercetin 7-*O*-glucoside from quercetin, along with the 3,7-*O*-diglucoside as a minor product. The conversion rate was 25–40% after 15-h incubation with the substrate (Willits et al. 2004). Several additional UGTs from *Arabidopsis*, with amino acid identities to UGT71G1 from 17% to 46%, also converted quercetin aglycone to different glycosides in *E. coli*. Mono or diglucosides were produced with yields from 0.19- to 10.90-mg/L culture (Lim et al. 2004). In our study, 17–20 mg/L quercetin monoglucoside was obtained. The glycosides produced in the cell culture system were also reported to release to the medium (Lim et al. 2004; Willits et al. 2004). Although the mechanisms of aglycone uptake and glycoside secretion by the bacteria are not known, several efflux pump systems have been reported suggesting that the uptake and secretion of small molecules are active processing (Kruse et al. 2002; Livshits et al. 2003; Van Dyk et al. 2004; Zakataeva et al. 1999). DMSO have been commonly used as a solvent of flavonoid substrates in enzyme activity assay in vitro or in cell culture feeding studies in vivo. Our studies showed that the bacteria continued to grow to high density during the time course and the soluble enzyme were well expressed suggesting that the DMSO concentration we used (0.05%) did not have significant toxicity to the cells.

In the present paper, we extended our study to evaluate the utilization of two structure-directed mutants of UGT71G1 for glycosylation activity in *E. coli*. The Tyr202Ala mutant produced higher amounts of both quercetin and kaempferol glucosides than did the Phe148Val mutant. Amino acids Phe148 and Tyr202 are located at one end of the acceptor binding pocket and are close to each other in the three-dimensional structure of UGT71G1 (Shao et al. 2005). These mutations would reduce the size of the amino acid and, in turn, increase the volume of the binding pocket and allow the 3-hydroxyl of the substrate to move closer to the C1 reaction center on the UDP-glucose for favorable glycosylation. Mutations of Phe148 to valine or Tyr202 to alanine change the regiospecificity for quercetin glycosyl-ation from predominantly 3'-OH to 3-OH (He et al. 2006). Our study showed that these structure-directed mutants can be used in vivo in *E. coli* to regioselectively synthesize small molecule glucosides. Advances in our understanding

of structure–activity relationships for plant small molecule glycosyltransferases will provide more opportunities for the design of novel catalysts to produce bioactive glycosides.

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