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Metabolic engineering of *Escherichia coli* for the biosynthesis of flavonoid-*O*-glucuronides and flavonoid-*O*-galactoside

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Abstract Most flavonoids are glycosylated and the nature of the attached sugar can strongly affect their physiological properties. Although many flavonoid glycosides have been synthesized in *Escherichia coli*, most of them are glucosylated. In order to synthesize flavonoids attached to alternate sugars such as glucuronic acid and galactoside, *E. coli* was genetically modified to express a uridine diphosphate (UDP)-dependent glycosyltransferase (UGT) specific for UDP-glucuronic acid (AmUGT10 from *Antirrhinum majus* or VvUGT from *Vitis vinifera*) and UDP-galactoside (PhUGT from *Petunia hybrid*) along with the appropriate nucleotide biosynthetic genes to enable simultaneous production of their substrates, UDP-glucuronic acid and UDP-galactose. To engineer UDP-glucuronic acid biosynthesis, the *araA* gene encoding UDP-4-deoxy-4-formamido-L-arabinose formyltransferase/UDP-glucuronic acid C-4″ decarboxylase, which also used UDP-glucuronic acid as a substrate, was deleted in *E. coli*, and UDP-glucose dehydrogenase (*ugd*) gene was overexpressed to increase biosynthesis of UDP-glucuronic acid. Using these strategies, luteolin-7-*O*-glucuronide and quercetin-3-*O*-glucuronide were biosynthesized to levels of 300 and 687 mg/L, respectively. For the synthesis of quercetin 3-*O*-galactoside, *UGE* (encoding UDP-glucose epimerase from *Oryza sativa*) was overexpressed along with a glycosyltransferase specific for quercetin and UDP-galactose. Using this approach, quercetin 3-*O*-galactoside was successfully synthesized to a level of 280 mg/L.

Keywords Flavonoid-*O*-glucuronide · Flavonoid-*O*-galactoside · Metabolic engineering · UGT

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Introduction

Flavonoids are plant secondary metabolites derived from the amino acids, phenylalanine, and tyrosine via the phenylpropanoid pathway (Winkel-Shirely 2001). Most flavonoids exist as *O*-glycosylated forms, but some plants produce *C*-glycosylated flavonoids (Bowles et al. 2006). *O*-Glycosylation reactions occur at hydroxyl groups during the final stage of flavonoid biosynthesis (Vogt and Jones 2001) while *C*-glycosylation occurs during flavonoid biosynthesis (Brazier-Hicks et al. 2009). Flavonoids can be glycosylated with various sugars. Sugars commonly linked to flavonoids include arabinose, galactose, glucose, rhamnose, glucuronic acid, and xylose (Bowles et al. 2006).

The biosynthesis of flavonoid-*O*-glycosides is mediated by nucleotide diphosphate-dependent glycosyltransferases (UGTs) (Vogt and Jones 2001), most of which use uridine diphosphate sugars as sugar donors, although thymidine derivatives can also be used (Lim et al. 2006). Thus, the biosynthesis of nucleotide sugars is a prerequisite to the formation of flavonoid-*O*-glycosides. Nucleotide sugar biosynthesis has been extensively studied in plants due to its role in cell wall biosynthesis (Seifert 2004). UDP-glucose is eventually converted into UDP-galactose, UDP-glucuronic acid, or UDP-rhamnose. UDP-glucuronic acid serves as a precursor to UDP-xylose, UDP-adipose, and UDP-arabinose. GDP-sugars such as GDP-mannose, GDP-galactose, GDP-fucose, and GDP-glucose are also found in plants. Most of the genes involved in nucleotide sugar biosynthesis in plants have been

characterized (Reiter 2008). Furthermore, genes regulating nucleotide sugar biosynthesis in plants and microorganisms have been successfully expressed in heterologous systems such as *Escherichia coli* and *Saccharomyces cerevisiae* (Oka and Jigami 2006; Yang et al. 2011). These systems serve as host that provide sugar donors for the synthesis of secondary metabolite-glycosides, including flavonoid glycosides.

The biosynthesis of phytochemicals in *E. coli* has been widely studied. The flavonoid biosynthesis pathway has been a good model for biosynthesis of phytochemicals in *E. coli* (Horinouchi 2008; Flower and Koffas 2009; Du et al. 2010). Simple modification reactions, semi-synthesis, and total synthesis have been performed by introducing flavonoid biosynthetic genes into *E. coli*. Although flavonoids have been successfully synthesized from glucose in *E. coli* (Santos et al. 2011; Kim et al. 2013a, b), there is an advantage to introducing simple modifications into flavonoids through biotransformation (Kim et al. 2005; 2006). Biotransformation of flavonoids using *E. coli* harboring UGT has been used successfully to produce flavonoid glucosides (Lim et al. 2006; Ko et al. 2006). *E. coli* provides the sugar donors (nucleotide sugars) necessary to synthesize flavonoid glycosides using exogenous flavonoids. *E. coli* synthesizes diverse nucleotide sugars for polysaccharide biosynthesis, which serve as sugar donors. To date, several flavonoid glycosides have been synthesized through biotransformation in *E. coli*. These include flavonoid-*O*-glucoside (Ko et al. 2006: Lim et al. 2004), flavonoid-*O*-xyloside (Pandey et al. 2013; Han et al. 2014), flavonoid-*O*-rhamnoside (Kim et al. 2012a; 2013a, b), flavonoid-*O*-(*N*-acetylglucosamine) (Kim et al. 2012b), flavonoid-*O*-(6-deoxytalose) (Yoon et al. 2012), and flavonoid-3-*O*-(4-deoxy-4-formamido-*L*-arabinose) (flavonoid-3-*O*-Ara4FN) (Kim et al. 2010). Although naringenin 7-*O*-glucuronide was synthesized previously, the final yields were not reported (Simkhada et al. 2010).

Flavonoid-*O*-galactoside and flavonoid-*O*-glucuronide, whose synthesis has not been extensively attempted in *E. coli*, have diverse biological activities. For example, quercetin-3-*O*-galactoside (hyperoside) has antidepressant effects (Zheng et al. 2012) and anti-inflammatory activity (Kim et al. 2011). Quercetin-3-*O*-glucuronide (miquelianin) also has antidepressant effects (Juergenliemk et al. 2003) and can prevent Alzheimer's disease and hypertension (Ho et al. 2013; Balasuriya and Rupasinghe 2012). Luteolin-7-*O*-glucuronide has anti-genotoxic and choleretic effects (Orhan et al. 2013; Benedek et al. 2006). In the present study, we synthesized flavonoid-*O*-glucuronide (luteolin-7-*O*-glucuronide and quercetin-3-*O*-glucuronide) and flavonoid-*O*-galactoside (quercetin-3-*O*-galactoside) in *E. coli*. We expressed specific UGTs for either UDP-glucuronic acid or UDP-galactose in *E. coli* and engineered its nucleotide sugar metabolism. Using this approach, we could achieve yield of more than 280 mg/L for flavonoid-*O*-galactoside and flavonoid-*O*-glucuronides.

Materials and methods

Nucleic acid manipulation

UGT from *Antirrhinum majus* (*AmUGT10*; Gene ID AB362988; Noguchi et al. 2009), which was used for the production of luteolin-7-*O*-glucuronide, was cloned using reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from the flower of *A. majus* using the Qiagen Plant Total RNA Isolation kit (Qiagen, Hilden, Germany). cDNA was synthesized using Omniscript reverse transcriptase with oligo dT as a primer. The primers for AmUGT10 are listed in Table 1. *AmUGT10* was subcloned into the *Eco*RI/*Not*I site of pETDuet-1 (Novagen), and the resulting construct was named pE-AmUGT (Table 1). To synthesize quercetin-3-*O*-galactoside, UGT from *Petunia hybrid* (PhUGT, GenBank ID; AF165148; Miller et al. 1999) was cloned by polymerase chain reaction (PCR) using cDNA as template. The resulting PCR product was digested with *Eco*RI/*Not*I and subcloned into the corresponding sites of pGEX 5X-3 (pG-PhUGT in Table 1). *OsUGE* (UDP-glucose epimerase from *Oryza sativa*; GenBank ID; NP_001062869.1) (Kim et al. 2009) was subcloned into the *Eco*RI/*Not*I site of pCDFDuet (pC-OsUGE in Table 1). UDP-glucose 4-epimerase (*galE*, GenBank ID; 945354) from *E. coli* DH5α was subcloned into the *Eco*RI/*Not*I site of pCDFDuet (pC-galE in Table 1). UGT from *Vitis vinifera* (VvUGT; GenBank ID XM_002280887; Ono et al., 2010), which was used for the production of quercetin-3-*O*-glucuronide, was cloned using RT-PCR. Total RNA was isolated from the leaves for *V. vinifera*, and cDNA was synthesized as described above. The PCR product was subcloned into the *Eco*RI/*Not*I site of pGEX 5X-3 (pG-VvUGT in Table 1). UDP-glucose dehydrogenase (*ugd*; Gene ID, 946571) from *E. coli* was cloned using PCR using *E. coli* genomic DNA as a template. The resulting PCR product was sequenced and subcloned into the *Nde*I/*Xho*I site of pE-AmUGT (pE-AmUGT-Ecugd in Table 1). *Ugds* from *Arabidopsis thaliana* (Gene ID, AT3G29360) and *Glycine max* (Gene ID, U53418) were cloned using RT-PCR. The resulting PCR products were subcloned into the *Nde*I/*Kpn*I site of pE-AmUGT for *AtUgd* (pE-AmUGT-AtUgd) and into the *Nde*I/*Xho*I site of pE-AmUGT for *GmUgd* (pE-AmUGT-GmUgd). Primers used are listed in Table 1.

Gene deletion in *E. coli* BL21 (DE3) was carried out using the Quick & Easy Conditional Knock Out Kit (Gene Bridges, Heidelberg, Germany). The *arnA* deletion mutant was developed as described in Kim et al. (2010).

Biotransformation of flavonoid using *E. coli*

An overnight culture of *E. coli* harboring specific genes was inoculated into 50 mL fresh of LB medium containing

Table 1 Plasmids and strains used in the present study

Plasmids or *E. coli* strain	Relevant properties or genetic marker	Source or reference
Plasmids		
pACYCDuet	P15A ori, Cm^r	Novagen
pCDFDuet	CloDE13 ori, Str^r	Novagen
pETDuet	f1 ori, Amp^r	Novagen
pGEX 5X-3	pBR322ori, Amp^r	GE healthcare
pE-AmUGT	pET+*UGT10* from *Antirrhinum majus*	This study
pE-AmUGT-Ecugd	pET+*UGT10* from *A. majus*+*ugd* from *E. coli*	This study
pE-Amugt-Atugd	pET+*UGT10* from *A. majus*+*ugd* from *A. thalians*	This study
pE-Amugt-Gmugd	pET+*UGT10* from *A. majus*+*ugd* from *Glycine max*	This study
pG-PhUGT	pGEX 5X-3+*UGT* from *Petunia hybrid*	This study
pG-VvUGT	pGEX 5X-3+*UGT* from *Vitis vinifera*	This study
pA-Ecugd	pACYC+*ugd* from *E. coli*	This study
pC-OsUGE	pCDF+*UGE* from *Oryza sativa*	Kim et al. (2009)
pC-galE	pCDF+*galE* from *E. coli*	This study
Strains		
BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm lon* (DE3)	Novagen
B-arnA	BL21 (DE3) *ΔarnA*	Kim et al. (2010)
B500	BL21 harboring pE-AmUGT	This study
B501	BL21 harboring pE-AmUGT-Ecugd	This study
B502	BL21 harboring pE-AmUGT-Atugd	This study
B503	BL21 harboring pE-AmUGT-Gmugd	This study
B504	BarnA harboring pE-AmUGT	This study
B505	BarnA harboring pE-AmUGT-Ecugd	This study
B506	BarnA harboring pE-AmUGT-Atugd	This study
B507	BarnA harboring pE-AmUGT-Gmugd	This study
B508	BL21 harboring pG-VvUGT	This study
B509	BarnA harboring pG-VvUGT and pA-Ecugd	This study
B510	BL21 harboring pG-PhUGT and pC-galE	This study
B511	BL21 harboring pG-PhUGT and pC-OsUXE	This study
Primers		
AmUGT10 forward	ATGAATTCGATGGAGGACACTATCGTTCTC	AB362988
AmUGT10 reverse	CATGCGGCCGCTTAAGAAACCACCATATCA	AB362988
PhUGT forward	ATGGAATTCGATGTCCAATTACCATGTTGC	AF165148
PhUGT reverse	CATGCGGCCGCTTAATTGCAAGAGGTGATA	AF165148
VvUGT forward	ATGAATTCGATGGCTGAAAAACCTCCCCAC	XM_002280887
VvUGT reverse	ATGCGGCCGCCTAATAATTCTTGTGAGCCTTCC	XM_002280887
Atugd forward	AACATATGGTGAAGATATGTTGTAT	AT3G29360
Atugd reverse	AAGGTACCTTAGGCAACGGCAGGCATGT	AT3G29360
Gmugd forward	AACATATGgtgaagatttgctgcat	U53418
Gmugd reverse	AACTCGAGTTATGCCACAGCAGGCATGT	U53418

Restriction enzyme sites were underlined

appropriate antibiotics. The cells were grown until the OD_{600} reached 0.8. Genes in the *E. coli* expression vector were induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The transformants were grown for an additional 20 h at 18 °C. Cells were harvested by centrifugation and resuspended in fresh M9 medium containing 2 % glucose and antibiotics and 1 mM IPTG. To compare the production of luteolin-7-*O*-glucuronide among different *E. coli* strains (B500-B507, Table 1), the cell density was adjusted to an OD_{600} value of 2. The total reaction volume was 2 mL in a test tube (14 mm×145 mm). One hundred micromoles of luteolin was added, and the reaction

mixture was incubated at 25 °C for 1.5 h. Then, an additional 100 μM luteolin was added, and the mixture was incubated for an additional 1.5 h. To determine the optimum cell concentration, 200 μM of luteolin was added. After 2 h, an additional 200 μM luteolin was added, and the reaction mixture was incubated for 2 h. To compare the production of quercetin-3-*O*-glucuronide among different *E. coli* strains, the cell densities were adjusted to an OD_{600} value of 3, and the reactions were carried out for 2 h after addition of 200 μM quercetin.

The effect of *EcgalE* and *OsUGE* on the production of quercetin-3-*O*-galactoside in *E. coli* BL21 (DE3) was tested using strains B510 and B511 (Table 1). Each strain was cultured as described above. One hundred micromoles of quercetin was added, and the mixture was incubated at 30 °C for 1.5 h with shaking.

To analyze quercetin-3-*O*-galactoside, the culture supernatant was harvested, extracted twice with an equal volume of ethyl acetate and dried in a vacuum. The dried sample was dissolved in dimethyl sulfoxide (DMSO) and analyzed by high-performance liquid chromatography (HPLC) using a Varian HPLC equipped with a photodiode array (PDA) detector and a Varian C18 reverse-phase column (Varian, 4.60× 250 mm, 3.5 μm particle size). Cell growth was monitored by determining the absorbance at 600 nm. For analysis of flavonoid-*O*-glucuronic acid, the reaction supernatant was boiled for 3 min and centrifuged for 10 min at 13,000*g*. The supernatant was analyzed with HPLC.

The mean and standard error of the mean were calculated from three experiments. Analysis of variance (ANOVA) was carried out using Tukey's method with a significance level of $P=0.01$. Statistical analyses were performed using Microsoft Office Excel 2010.

The molecular masses of the reaction products were determined by mass spectrometry as described previously (Lee et al. 2007). The structures of the reaction products were determined using nuclear magnetic resonance (NMR) spectroscopy as described previously (Kim et al. 2004). The following data were obtained. Quercetin-3-*O*-galactoside: ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.67 (dd, J=8.5, 2.2 Hz, 1H, H-6′), 7.54 (d, J=2.2 Hz, 1H, H-2′), 6.83 (d, J= 8.5 Hz, 1H, H-5′), 6.41 (d, J=2.0 Hz, 1H, H-8), 6.21 (d, J= 2.0 Hz, 1H, H-6), 5.38 (d, J=7.7 Hz, 1H, H-1″), 3.66 (d, J= 3.1 Hz, 1H, H-4″), 3.58 (m, 1H, H-2″), 3.47 (dd, J=9.6, 5.2 Hz, 1H, H-6″), 3.39 (dd, J=9.5, 3.3 Hz, 1H, H-3″), 3.33 (d, J=2.9 Hz, 1H, H-5″), 3.31 (m, 1H, H-6″); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 177.4 (C-4), 164.2 (C-7), 161.2 (C-5), 156.3 (C-2), 156.2 (C-9), 148.4 (C-4′), 144.8 (C-3′), 133.4 (C-3), 121.1 (C-1′), 122.0 (C-6′), 115.9 (C-2′), 115.2 (C-5′), 103.9 (C-10), 101.8 (C-1″), 98.7 (C-6), 93.5 (C-8), 75.8 (C-5″), 73.2 (C-3″), 71.2 (C-2″), 67.9 (C-4″), 60.1 (C-6″). Quercetin-3-*O*-glucuronic acid: ^{1}H NMR (CD_3OD, 400 MHz): δ 7.61–7.65 (m, 2H), 6.85 (d, J= 8.4 Hz, 1H), 6.39 (d, J=2.0 Hz, 1H), 6.20 (d, J=2.1 Hz, 1H), 5.34 (d, J=7.6 Hz, 1H), 3.74 (d, J=9.7 Hz, 1H), 3.60 (d, J=8.8 Hz, 1H), 3.56 (d, J=3.1 Hz, 1H), 3.45–3.53 (m, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ 178.7, 165.4, 162.4, 158.4, 157.9, 149.3, 148.3, 145.4, 134.8, 122.8, 122.2, 116.6, 115.4, 105.0, 103.6, 99.3, 94.1, 77.0, 76.6 74.8, 72.3. Luteolin-7-*O*-glucuronic acid: ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.44 (d, J=8.0 Hz, 1H), 7.43 (s, 1H), 6.90 (d, J= 8.0 Hz, 1H), 6.81 (d, J=2.0 Hz, 1H), 6.75 (s, 1H), 6.45 (d, J= 2.0 Hz, 1H), 5.26 (d, J=6.8 Hz, 1H), 4.00 (d, J=9.2 Hz, 1H), 3.38 (t, J=9.2 Hz, 1H), 3.33 (t, J=9.0 Hz, 1H), 3.29 (t, J= 7.2 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 181.85, 170.38, 164.45, 162.49, 161.10, 156.91, 149.92, 145.75, 121.27, 119.12, 115.96, 113.49, 105.37, 103.10, 99.35, 99.13, 94.48, 75.65, 75.15, 72.74, 71.29.

Results

Production of flavonoid-*O*-glucuronides

We planned to use *E. coli* to synthesize flavonoid glucuronide. *E. coli* synthesizes UDP-glucuronic acid but does not contain a UGT specific for luteolin and UDP-glucuronic acid. AmUGT10 uses UDP-glucuronic acid as a sugar donor and luteolin as the optimal sugar acceptor to make luteolin-7-*O*-glucuronide (Noguchi et al. 2009). *E. coli* harboring pE-AmUGT (B500 in Table 1) was used for biotransformation of luteolin, and analysis of the reaction product with HPLC showed a new peak at 462 Da (Fig. 1a), whose molecular mass was consistent with that of a glucuronic acid attached to luteolin (Fig. 1d). In addition, the reaction product differed in retention time from luteolin-7-*O*-glucoside (Fig. 1c). MS/MS analysis of the reaction product revealed a compound with a molecular mass of 286 Da (Fig. 1d), which corresponded to the molecular weight of luteolin. Thus, we concluded that *E. coli* harboring pE-AmUGT synthesized luteolin-7-*O*-glucuronic acid from luteolin. Furthermore, NMR revealed the structure of the biotransformation product to be luteolin-7-*O*-glucuronide (see "Materials and methods").

To increase the production of luteolin-7-*O*-glucuronide, it was necessary to increase the supply of UDP-glucuronic acid in *E. coli* because luteolin was supplied exogenously, but UDP-glucuronic acid was synthesized by the *E. coli* itself. UDP-glucuronic acid is synthesized from UDP-glucose by UDP-glucose dehydrogenase (ugd) and is then converted into UDP-4″-ketose by UDP-L-Ara4N formyltransferase/UDP-GlcA C-4″-decarboxylase (arnA), which is involved in the biosynthesis of outer membrane lipopolysaccharides (Breazeale et al. 2005). Thus, in order to increase the supply of UDP-glucuronic acid, *arnA* was first deleted in *E. coli* (B-araA in Table 1), and B-arnA was transformed with pE-

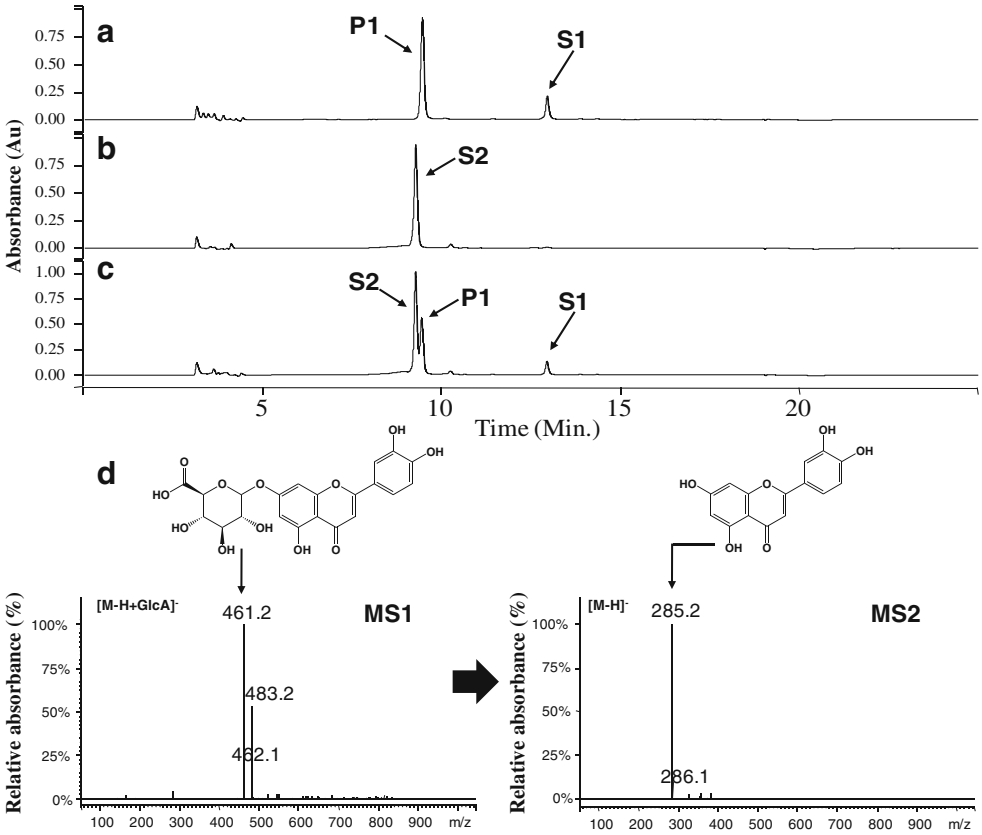

Fig. 1 **a** HPLC profile of the reaction product (P1) obtained from *E. coli* strain B500 supplemented with luteolin (S1); **b** luteolin-7-*O*-glucose standard (S2); **c** mixture of the reaction product and luteolin-7-*O*-glucose standard; **d** MS/MS analysis of the reaction product (P1). The mass spectrometer was operated in negative mode

AmUGT. The resulting strain B504 produced 31.1 mg/L of luteolin-7-*O*-glucuronide, which is approximately 121 % more than B500 (14.1 mg/L) (Fig. 2).

In order to increase the supply of UDP-glucuronic acid, *ugd*, which encodes a protein converting UDP-glucose to UDP-glucuronic acid, was overexpressed in *E. coli*. Three *ugd* genes from *E. coli*, *A. thaliana*, and *G. max* were tested in two different *E. coli* strains (wild type and B-araA). The product of biotransformation of luteolin for each strain (B500~B507 in Table 1) was analyzed using HPLC. *E. coli* transformants containing *ugd* produced more luteolin-7-*O*-glucuronide than *E. coli* transformants not containing *ugd*. The *ugd* of *E. coli* (Ecugd) was the most efficient followed by those of *A. thaliana* and *G. max* (Fig. 2). Strain B505 (B-araA harboring pE-AmUGT-Ecugd) produced 197 % more luteolin-7-*O*-glucuronide (92 mg/L) than strain B504 (B-araA harboring only pE-AmUGT, 31.1 mg/L). Thus, an increased supply of UDP-glucuronic acid by *ugd* overexpression is required to increase the production of luteolin-7-*O*-glucuronide. As shown above, the B-araA strain produced more luteolin-7-*O*-glucuronide than BL21 for all constructs. B500, in which only *AmUGT* was overexpressed in wild type *E. coli* BL21 (DE3), synthesized approximately 14 mg/L luteolin-7-*O*-glucuronic acid while B505, in which both AmUGT and Ecugd were overexpressed in B-araA, produced approximately 92 mg/L of luteolin-7-*O*-glucuronide.

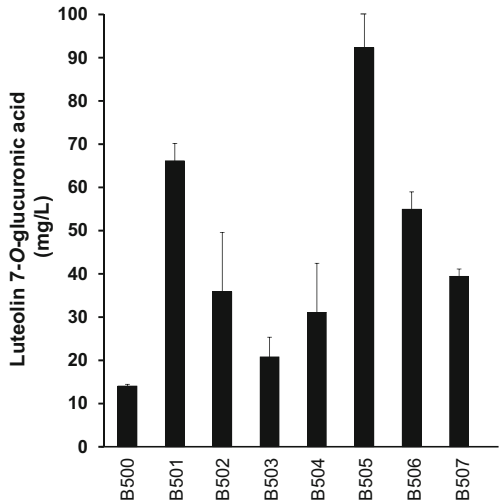

Fig. 2 Comparison of luteolin-7-*O*-glucuronide production from luteolin using different combinations of *ugd* and *E. coli* mutant strains. Data are mean values±SD from three independent experiments

We employed another UGT (VvUGT) to synthesize quercetin-3-*O*-glucuronide from quercetin in *E. coli*. Strain B508 (Table 1) was used for biotransformation of quercetin, and the biotransformation product was analyzed. A new peak was observed at 478 Da (Fig. 3a), the molecular mass corresponding to that of quercetin-3-*O*-glucuronide. The structure of the reaction product was determined to be quercetin-3-*O*-

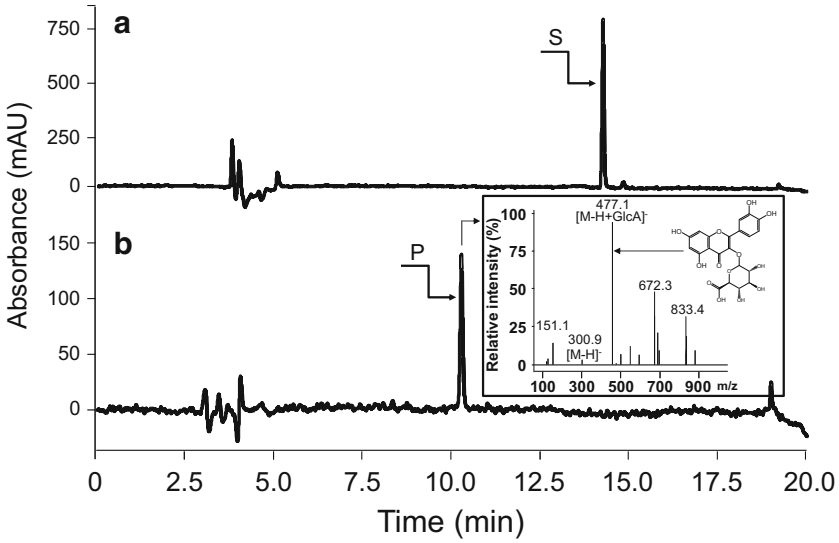

Fig. 3 HPLC analysis of the reaction product obtained with strain B508 supplemented with quercetin as a substrate. **a** The reaction product obtained with *E. coli* BL21 harboring empty vector, 5X-3; **b** HPLC profile and MS analysis of the reaction product obtained with strain B508. The mass spectrometer was operated in negative mode

glucuronide by NMR (see "Materials and methods"). Similar to the synthesis of luteolin-7-*O*-glucuronide, the production of quercetin-3-*O*-glucuronide increased in the *araA* deletion strain (B-araA) and overexpressing *Ecugd*. Strain B508 (*E. coli* BL21 [DE3] harboring pG-VvUGT) produced approximately 38 mg/L quercetin-3-*O*-glucuronide, while strain B509 (B-ara harboring pG-VvUGT and pA-Ecugd) produced approximately 62 mg/L quercetin-3-*O*-glucuronide. Therefore, the engineered *E. coli* strain produced approximately 1.6-fold as much quercetin-3-*O*-glucuronide as wild type *E. coli* strain.

The production of luteolin-7-*O*-glucuronide was optimized with strain B505. First, we determined the optimal cell density. After induction, the cell density was adjusted to OD_{600} values of 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4, 5, and 7. Initially, 200 μM (57.2 mg/L) luteolin was added. After incubation of the reaction mixture at 25 °C for 2 h, an additional 200 μM of luteolin was added, and the reaction mixture was incubated 25 °C for 6 h. In the reaction mixture containing cells at an OD_{600} value of 3.5, 320 μM (148 mg/L) luteolin-7-*O*-glucuronide was produced, which was the highest yield among the cell densities tested. The production of luteolin-7-*O*-glucuronide was monitored. The cell density of strain B505 was 3.5 at 600 nm. One hundred micromolar of luteolin (28.6 mg/L) was added at 0, 2, 4, 6, 8, 10, and 12 h (the final concentration was 700 μM [200.4 mg/L]) because the solubility of luteolin is low and a high concentration might cause cell lysis. The mixture was incubated at 25 °C for 48 h. The reaction mixture was collected periodically and analyzed by HPLC. The production of luteolin-7-*O*-glucuronide continued to increase until 24 h. At this point, 300.5 mg/L of luteolin-7-*O*-glucuronide (approximately 650 μM) was produced (Fig. 4a). The conversion yield from luteolin to luteolin-7-*O*-glucuronide was 92.8 %.

The production of quercetin-3-*O*-glucuronide was also monitored for 48 h in strain B509. Quercetin (100 μM=30.2 mg/L) was added every hour up to 12 h and then at 24 h. Therefore, the final amount of quercetin added was 1.4 mM (422.8 mg/L). The initial cell density was set to an OD_{600} value of 3. The production of quercetin-3-*O*-glucuronide increased rapidly for 12 h, and then the rate of increase slowed. After 48 h, 659.6 mg/L of quercetin-3-*O*-glucuronide (1.38 mM) was produced (Fig. 4b). The conversion yield was 98.5 %.

Production of quercetin-3-*O*-galactoside

PhUGT, flavonol-3-*O*-galactosyltransferase, was used to produce quercetin-3-*O*-galactoside (Miller et al. 1999). *E. coli* BL21 (DE3), which is a generally used host for expressing foreign genes that was transformed with pG-PhUGT (Table 1). However, *galE*, which is a UDP-glucose 4-epimerase and catalyzes the interconversion of UDP-glucose and UDP-galactose, is absent in *E. coli* BL21 (DE3), and this strain no longer synthesizes UDP-galactose. Therefore, biotransformation of quercetin using *E. coli* BL21 (DE3) harboring pG-PhUGT did not produce any reaction product (Fig. 5a). It clearly showed that UDP-galactose is absent from *E. coli* BL21 (DE3) due to the absence of *galE*. On the other hand, *E. coli* DH5α harboring pG-PhUGT produced a new reaction product from quercetin, which was predicted to be quercetin-3-*O*-galactoside, because *E. coli* DH5α contains an intact copy of *galE*. Thus, both *galE* and *PhUGT* are required to synthesize quercetin-3-*O*-galactoside in *E. coli* BL21 (DE3). We decided to use *E. coli* BL21 (DE3) instead of DH5α because this strain is commonly used to express foreign genes.

In order to provide UDP-galactose to *E. coli* BL21, two genes, *EcgalE* and *OsUGE*, were tested. Both EcgalE and OsUGE interconvert UDP-glucose and UDP-galactose.

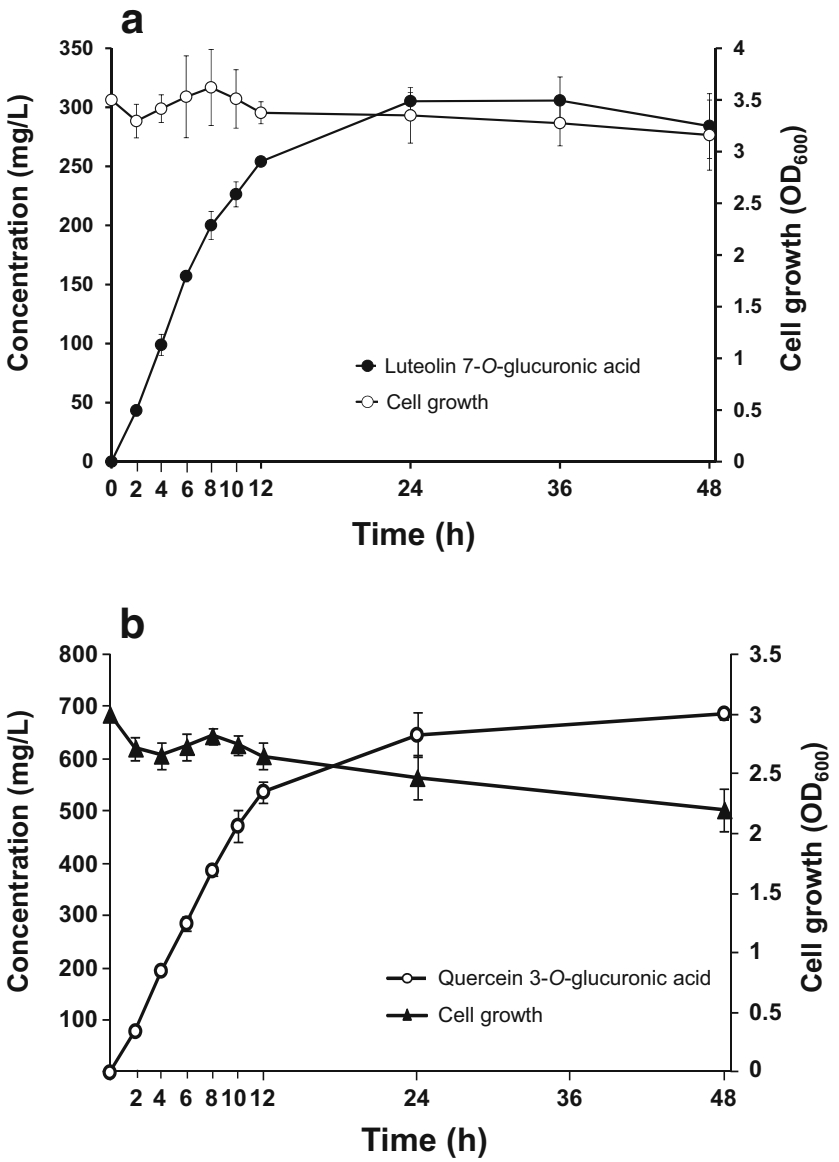

Fig. 4 Production of luteolin-7-*O*-glucuronide using strain B505 (**a**) and quercetin-3-*O*-glucuronide using B509 (**b**). Data are mean values±SD from three independent experiments. For the synthesis of luteolin-7-*O*-glucuronide, 100 μM luteolin was added at 0, 2, 4, 6, 8, 10, and 12 h (total 700 μM luteolin was added). For the synthesis of quercetin-3-*O*-galactoside, 200 μM quercetin was added every 2 h up to 12 h and then at 24 h (total 1.4 mM quercetin was added). Sample was taken at each time point, and the product was analyzed using HPLC

E. coli BL21 (DE3) was transformed with pC-galE or pC-OsUGE along with pG-PhUGT (Table 1). The production of quercetin-3-*O*-galactoside in strains, B510 and B511 (Table 1) were compared. Strain B511 produced 38 mg/L quercetin-3-*O*-galactoside, which was 1.9-fold as much as that produced by strain B510 (20 mg/L). This indicated that OsUGE more effectively converted UDP-glucose to UDP-galactose. The structure of the biotransformation product was determined to be quercetin-3-*O*-galactoside by NMR (see "Materials and methods").

Using strain B511, we monitored the production of quercetin-3-*O*-galactoside. The initial cell density was set to OD_{600} value of 3. Quercetin (100 μM=30.2 mg/L) was added at 0, 3, 6, 9, 13, 24, and 36 h (total 700 μM [211.5 mg/L] quercetin was added). Quercetin-3-*O*-galactoside was rapidly produced until 24 h. After 36 h, its production did not increase substantially. Approximately 280.4 mg/L quercetin-3-*O*-galatoside (approximately 603 μM) was produced after 60 h of biotransformation (Fig. 6). The conversion yield was 86.1 %.

Discussion

UDP-glucose is a substrate for the synthesis of UDP-galactose, UDP-glucuronic acid, and UDP-Ara4FN in *E. coli*. UDP-galactose is not synthesized in *E. coli* BL21 (DE3) because it lacks *galE*, which encodes the enzyme that converts UDP-glucose into UDP-galactose. Introducing *galE* into *E. coli* BL21 (DE3) resulted in the production of UDP-galactose, which served as a substrate for the production of

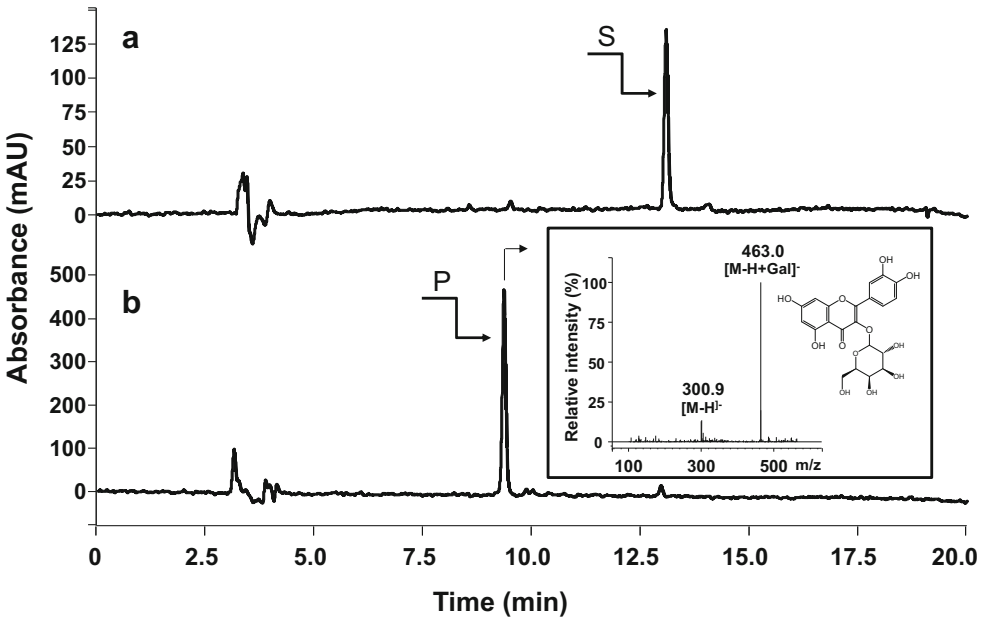

Fig. 5 HPLC profile of the reaction product obtained with strain B511 supplemented with quercetin as a substrate. **a** Reaction product obtained with *E. coli* BL21 harboring PhUGT; **b** HPLC profile and MS analysis of the reaction product obtained from strain B510 supplemented with quercetin as a substrate. The mass spectrometer was operated in negative mode

quercetin 3-*O*-galactoside. For the production of flavonoid-*O*-glucuronide, we also overexpressed *ugd* to enhance the conversion of UDP-glucose to UDP-glucuronic acid. This increased in luteolin-7-*O*-glucuronic acid production from 14 to 65 mg/L. Furthermore, an *E. coli* strain in which a gene (*arnA*) for a protein converting UDP-glucuronic acid to UDP-Ara4FN produced more luteolin-7-*O*-glucuronic acid (30 mg/L) than wild type (14 mg/L). Finally, *E. coli* strain, in which *ugd* was overexpressed and *arnA* was deleted, was the most effective for the production of luteolin-7-*O*-glucuronic acid (92 mg/L).

Two flavonoid-*O*-glucuronides (luteolin-7-*O*-glucuronide and quercetin-3-*O*-glucuronide) and quercetin-3-*O*-galactoside were synthesized in *E. coli* via biotransformation of exogenously supplied luteolin and quercetin, respectively. The UGTs that used (namely, AmUGT10, VvUGT, and PhUGT) have both sugar donor specificity and acceptor specificity (Miller et al. 1999; Noguchi et al. 2009; Ono et al., 2010). In regard to sugar acceptors, AmUGT10 could use flavonoids having a 7-hydroxy group such as naringenin, apigenin, and quercetin. Therefore, it is possible to make naringenin-7-*O*-glucuronide, apigenin-7-*O*-glucuronide, and quercetin-7-*O*-glucuronide. Kaempferol, which also has a 3-hydroxy group, serves as a substrate for PhUGT and VvUGT for the synthesis of kaempferol-3-*O*-galactoside and kaempferol-3-*O*-glucuronide, respectively.

Flavonoid-*O*-glucuronides and flavonoid-*O*-galactose have not been synthesized previously in *E. coli*. Most research on flavonoid glycosylation has been about the regioselective glucosylation of flavonoids, although glucuronylation and

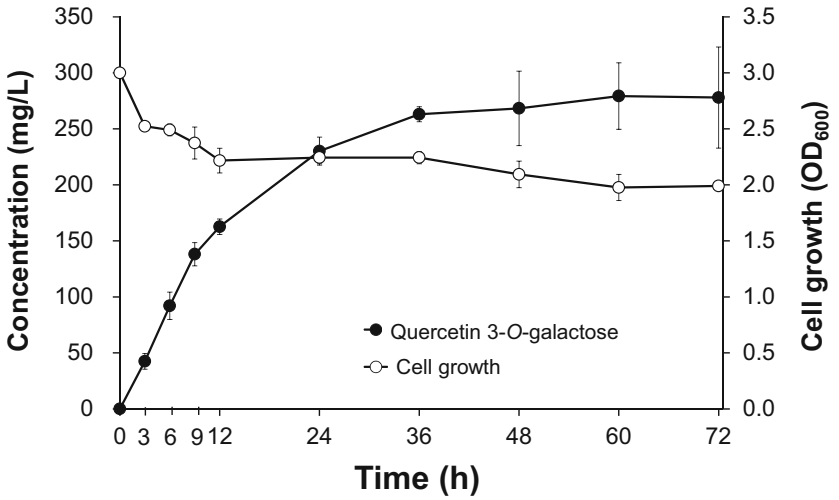

Fig. 6 Production of quercetin-3-*O*-galactoside from strain B511. Data are mean values±SD from three independent experiments. Quercetin (100 μM) was added at 0, 3, 6, 9, 12, 24, and 36 h (total 700 μM was added)

galactosylation of flavonoids in plants are also common (Xiao et al. 2014). The reason that glucosylation of flavonoids is common is because most UGTs use UDP-glucose as a sugar donor. In this study, we chose UGTs that use either UDP-glucuronic acid or UDP-galatose as a sugar donor. In addition, we engineered *E. coli* to provide higher levels of the appropriate sugar donors to enhance synthesis of flavonoid-*O*-glucuronides and flavonoid-*O*-galactosides. The *E. coli* strains which nucleotide biosynthesis pathways have been engineered could be used to attach glucuronic acid and galactose to other secondary metabolites.

Flavonoid glucosides were the most prevalent flavonoid glycosides synthesized using biotransformation. The final yield of quercetin- *O*-glucoside ranged from 1 to 10 mg/L (Lim et al. 2004). Using a controlled fermentation system, yields of approximately 100 mg/L were obtained (Lim et al. 2004). *E. coli* harboring isoflavone glucosyltransferase gave yields ranging from 10 to 20 mg/L genistein-7-*O*-glucoside or biochanin A-7-*O*-glucoside (He et al. 2008). These studies were performed using endogenous UDP-glucose as a substrate donor. In this study, 300 mg/L of luteolin-7-*O*-glucuronide, 687 mg/L of quercetin-3-*O*-glucuronide, and 280 mg/L of quercetin-3-*O*-galactoside were obtained This was achieved by engineering the nucleotide pathway of *E. coli*. Quercetin-3-*O*-rhamnoside and kaempferol-3-*O*-rhamnoside were also synthesized (150 to 200 mg/L) in *E. coli* strain with an engineered nucleotide synthesis pathway (Kim et al. 2012a, b). Thus, engineering the nucleotide pathway in *E. coli* is critical to increasing the yield of flavonoid glycosides.

We tested functionally identical genes from different organisms in order to identify the one with the highest productivity. For the synthesis of luteolin-3-*O*-glucuronide, *ugd* from *E. coli* was more effective than those from plants such as *A. thaliana* and *G. max*. On the other hand, Os*UGE* from rice was more effective than that from *E. coli* in the synthesis of quercetin-3-*O*-galactoside. Although we did not measure in vitro catalytic efficiency of the individual enzymes encoded by each gene, the most efficient enzyme was selected by comparing the final yield. Furthermore, in vitro catalytic efficiency is always not correlated with in vivo biotransformation efficiency (Lim et al. 2011). Thus, the overall balance of several reaction intermediates and the concentration of enzymes must be modulated in order to maximize the productivity.

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