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Molecules and Cells

Metabolic Engineering of *Escherichia coli* for the Biological Synthesis of 7-*O*-Xylosyl Naringenin

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Flavonoids are a group of polyphenolic compounds that have been recognized as important due to their physiological and pharmacological roles and their health benefits. Glycosylation of flavonoids has a wide range of effects on flavonoid solubility, stability, and bioavailability. We previously generated the *E. coli* BL21 (DE3) Δ*pgi* host by deleting the glucose-phosphate isomerase (Pgi) gene in *E. coli* BL21 (DE3). This host was further engineered for whole-cell biotransformation by integration of *galU* from *E. coli K12*, and expression of *calS8* (UDP-glucose dehydrogenase) and *calS9* (UDP-glucuronic acid decarboxylase) from *Micromonospora echinospora* spp. *calichensis* and *arGt-4* (7-*O*-glycosyltransferase) from *Arabidopsis thaliana* to form *E. coli* (US89Gt-4), which is expected to produce glycosylated flavonoids. To test the designed system, the engineered host was fed with naringenin as a substrate, and naringenin 7-*O*-xyloside, a glycosylated naringenin product, was detected. Product was verified by HPLC-LC/MS and ESI-MS/MS analyses. The reconstructed host can be applied for the production of various classes of glycosylated flavonoids.

INTRODUCTION

Flavonoids are aromatic secondary plant metabolites that have been recognized as important due to their physiological (Buslig and Manthey, 2002) and pharmacological roles and their health benefits (Valenzuela et al., 2003). Flavonoids show strong antioxidant and radical scavenging activity (Cao et al., 1997) and appear to be associated with reduced risk for certain chronic diseases (Kris-Etherton et al., 2004), the prevention of some cardiovascular disorders (Yochum et al., 1999) and certain kinds of cancerous processes (Kris-Etherton et al., 2002). Flavonoids also exhibit antiviral (Asres et al., 2005), antimicrobial (Cushnie and Lamb, 2005), and anti-inflammatory activities (Kim et al., 2004), beneficial effects on capillary fragility (Benavente-García et al., 1997), embryonic lethality (Lee, et al., 2008), an ability to inhibit human platelet aggregation (Tijburg et al., 1997), and antiulcer (Borrelli and Izzo, 2000) and antiallergenic (Middleton and Kandaswami, 1992) properties.

Glycosylation is a prominent modification reaction in plant metabolism. The targets of this modification are often secondary metabolites. The flavonoid aglycones, which have a variety of glycosylation sites, are converted into glycones by glycosyltransferase (GTs) in the last step of flavonoid biosynthesis. Flavonoids are stored in glycone form (Brugliera et al., 1994; Hu and Walker, 2002). In many cases, glycosylation is the last step in the biosynthesis of secondary plant products such as flavonoids, cyanohydrins, steroidal alkaloids and saponins. More than 6,000 different glucosidic derivatives have already been identified and described (Buslig and Manthey, 2002). Glycosylation mainly occurs on oxygen (COOH and OH), sulfur, nitrogen and carbon atoms with nucleotide-activated sugar acting as a donor substrate (Manach and Donovan, 2004). The transfer of a nucleotide-diphosphate activated monosaccharide unit (glucose, rhamnose, galactose, xylose, rutinose, and neohesperidose) (Valenzuela et al., 2003) to an acceptor molecule is catalyzed by glycosyltransferases (UGTs).

Among the diverse flavonoids, naringenin is a common starting material for biosynthesis of more complicated flavonoids. Hydroxylation, formation of double bond, methylation, glycosylation, and mannosylation are involved in further processing of naringenin (Tahara, 2007). Because of the biological impact of flavonoids on humans, simple plant extracts might not satisfy the necessary requirements, and chemical synthesis involves complicated processes. To overcome these obstacles, we generated an *E. coli* system containing the sugar biosynthetic pathway and the glycosyltransferase gene from *Arabidopsis thaliana* for the production of xylosylated Naringenin.

MATERIALS AND METHODS

Escherichia coli XL1-Blue (MRF) (Stratagene, USA) was used as a host cell for recombinant plasmid preparation and DNA manipulation, whereas *E. coli* BL21 (DE3) Δ*pgi* was used for biotransformation of the recombinant. For the selection and maintenance of plasmids, *E. coli* was grown at 37°C in Luria-Bertani (LB) broth or on agar plates supplemented with the appropriate amounts of antibiotics when necessary (ampicillin up to 50 μg ml^{-1}, Kanamycin up to 50 μg ml^{-1} and Spectinomycin 50 μg ml^{-1}). pGEM-T easy vector (Promega, USA), pLOI2223 (Integration vector, National Bioresource Project NIG, Japan) and pET28a+, CDFDuet-1 (Novagen, Germany) were used as vectors for cloning of polymerase chain reaction (PCR) products, integration and expression of the genes (Table 1), respectively.

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Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source or references
Strains		
E. coli XL1 blue	General cloning host	Stratagene PBL company
E. coli Bl21(DE3)	Expression host	Stratagene PBL company
E. coli Bl21(DE3) Δ*pgi*	Expression host	Unpublished data
Plasmids		
pGEM®-T easy vector	*E. coli* general cloning vector, Amp^r	Promega (USA)
pCDFDuet-1	Expression vector, Sm^r	Novagen (Germany)
pLOI2223	*E. coli* integration vector, Amp^r	NBRP (NIG, Japan): *E. coli*
pET28a+	Expression vector, Km^r	Novagen (Germany)
pCDCalS89	Expression vector pCDFDuet-1 containing *calS9*	This work
pLOIGalU	Integration vector pLOI2223 containing *galU*	This work
pArGt-4	Expression vector pET28a+ containing *argt-4*	This work
cDNA	NCBI Acession Number	
ArGt-4	BT000754 (At4g34138)	RIKEN Bioresource center

Table 2. Oligonucleotide primers used for amplification of genes

Gene	Sequence (5′-3′)	Portion of gene	Restriction site	Vectors
S8D	AGCGGATCCCATCATGCCGTTCCTTCC	5′	*Bam*HI	CDFDuet-1
	AGCAAGCTTTCACCTTCCAATGCCGC	3′	*Hin*dIII	
S9D	AACCATATGCCCAGATCCCTGGTCACC	5′	*Nde*I	CDFDuet-1
	AGTCTCGAGCTACCTGACGACCAGTCC	3′	*Xho*I	
GalU	AGCGAATTCATGGCTGCCATTAATACG	5′	*Eco*RI	pLOI2223
	GCAGGATCCTTACTTCTTAATGCCCAT	3′	*Bam*HI	
ArGt-4	GAAGGATCCATGGGAACTCCTGTCGAA	5′	*Bam*HI	pET28a+
	GGCAAGCTTTTATACCTTCTCTTTTTGCA	3′	*Hin*dIII	

In order to express UDP-xylose in *E. coli*, the *calS8* (UDP-glucose dehydrogenase) and *calS9* (UDP-glucuronic acid decarboxylase) genes were amplified from the recombinant provided by Dr. Andreas Bechthold and cloned in CDFDuet-1 (Novagen, Germany). Uridylyltransferase gene was amplified from *E. coli* K12 and cloned in pLOI2223 for integration and overexpression in *E. coli* BL21 (DE3) Δ*pgi*. *E. coli* BL21 (DE3) Δ*pgi* was constructed by disruption of glucose phosphate isomerase (*pgi*) gene disrupted from the chromosome of *E. coli* BL21 (DE3) by λ red mediated Quick and Easy BAC Modification Kit (Gene Bridges) as recommended. Glycosyltransferase enzyme was amplified from the cDNA of the *Arabidopsis thaliana* glycosyltransferase gene (BT000754, At4g34138), which was purchased from the RIKEN Bioresource Centre (http://www.brc.riken.jp/inf/en/index.shtml), and cloned into pET28a+ for expression in an engineered *E. coli*. The detailed list of primers and recombinants is found in Table 2. The final host strain for the production was prepared and named *E. coli* (US89Gt-4).

E. coli (US89Gt-4) was used for the whole cell biocatalyst assay. This strain was grown on LB medium. When the A_{600} reading of the bacterial culture reached 0.7, 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added; after 30 min, 0.2 mM naringenin was added separately and the culture was continued for 60 h. Supernatant was extracted by ethyl acetate and cell pellets were extracted with 80% methanol. The extracted compounds were concentrated, dissolved in methanol, and used for further analysis. To analyze the activity of the whole-cell biocatalyst cultured in phosphate buffer, the culture was grown as described above. When the OD reading of the bacterial culture reached 0.7, 0.2 m*M* IPTG was added. After 24-h induction at 20°C, the cells were collected by centrifugation, washed with 100 m*M* phosphate buffer (pH 7.0), and resuspended in 50 ml of 100 m*M* phosphate buffer (pH 7.0) (A_{600} ~ 0.7). Naringenin (0.2 mM) was added to each flask separately and the culture flask was incubated for 36 h. Both the cells and culture medium were collected as described above and analyzed by HPLC, LC-MS and ESI/MS-MS. Reverse-phase HPLC analysis was performed with a C18 column (30105-254630, Thermo, ODS Hypersil; 4.6 × 250 mm; 5 μm diameter particle) and an isocratic gradient of 70% water (0.1% trifluoroacetic acid) and 30% acetonitrile at flow rate of 1 ml/min. The absorbance was recorded at 330 nm.

RESULTS AND DISCUSSION

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-sugars to the *in vitro* system. Although different approaches have been reported, including chemical methods, enzymatic synthesis, and regeneration using UDP-D-glucose pyrophosphorylase and pyrophosphatase for synthesis of nucleoside diphosphate sugars (Bowles et al., 2006; Kim et al., 2006; Ko et al., 2006), these approaches are either laborious, difficult, or

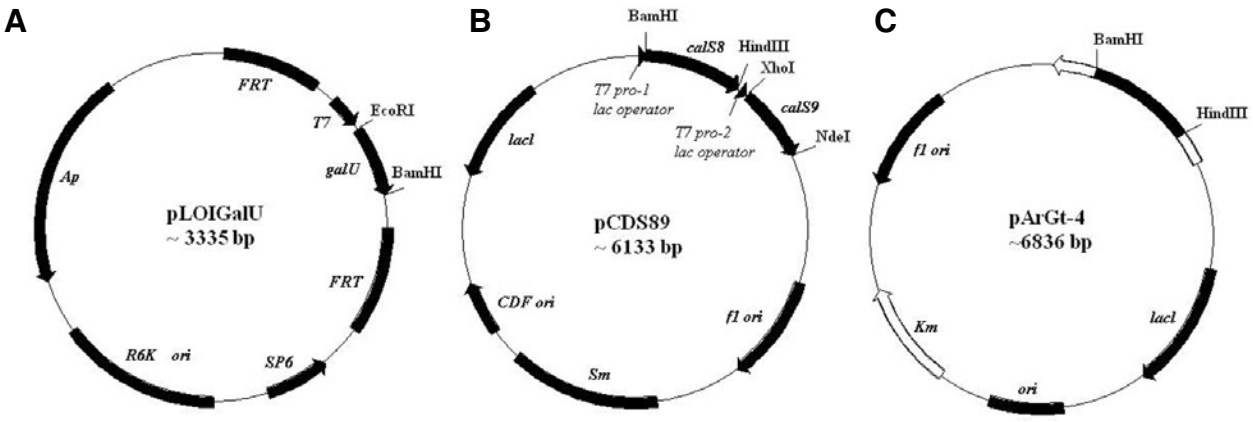

Fig. 1. (A) Recombinant plasmid of uridylyltransferase gene from *E. coli* K12 in integration vector pLOI2223 (B) *calS8* and *calS9* gene cloned in CDFDuet-1 vector (C) 7-*O*-glycosyltransferase cloned in pET28a+ expression vector to construct pArGt-4.

require sugar phosphates, phosphoenolpyruvate, and nucleotide 5′-triphosphate and are therefore costly. Since UDP-D-glucose is a natural intermediate in bacterial cell wall synthesis, using glycosyltransferase engineered in bacteria to synthesize small molecule glycosides should be an efficient approach to overcome the difficulties associated with the preparation of nucleotide sugars. Insertion of sugar cassettes with enzyme UDP-D-glucose dehydrogenase and UDP glucuronic acid decarboxylase also helps to produce flavonoid glycosylated with UDP-D-xylose. For this approach, we engineered *Escherichia coli* BL21 (DE3) to construct *E. coli* BL21 (DE3) Δ*pgi* because *Escherichia coli* BL21 (DE3) is a widely used expression host for many external enzymes. The intracellular D-glucose-6-phosphate (G-6-P) flux is utilized by diverse primary metabolic pathway enzymes. The crucial enzymes acting on G-6-P are glucose phosphate isomerase (Pgi), D-glucose-6-phosphate dehydrogenase (Zwf) and phosphoglucomutase (Pgm) encoded by the genes *pgi*, *zwf* and *pgm* respectively. These three enzymes have their respective roles in glycolysis, pentose phosphate and polysaccharide pathways. Disruption of one or more of the genes which are responsible to convert G-6-P into intermediate metabolites of the primary metabolic pathway can accumulate the pool of G-6-P (Fraenkel, 1968). Thus, an engineered strain, *E. coli* BL21 (DE3) Δ*pgi,* was constructed by disrupting glucose phosphate isomerase (*pgi*) gene from the chromosome (unpublished data) which was further engineered and applied as a host for the production of glycosylated flavonoids.

To increase the pool of UDP-D-glucose in the host, *galU* was constructed in the integration vector pLOI2223 (Fig. 1A) and transformed in *E. coli* BL21 (DE3) Δ*pgi*. Integration of *galU* allows accumulation of excessive UDP-D-glucose that is used to generate UDP-D-xylose after expression of UDP-glucose dehydrogenase (*calS8*) (Bililign et al., 2002) and UDP-glucuronic acid decarboxylase (*calS9*) (Simkhada et al., 2009) (Fig. 1B).

Glycosyltransferase typically transfers sugars onto lipophilic small molecule acceptors. The activated sugar donor for plant GTs is typically UDP-D-glucose, although UDP-D-rhamnose, UDP-D-galactose, UDP-D-xylose, and UDP-D-glucuronic acid have also been identified as activated sugars for the transfer reaction. For the glycosylation of UDP-D-xylose produced by the engineered host, *arGt-4* (7-*O*-glycosyltransferase) was cloned in pET28a+ (Fig. 1C) and transferred to the engineered host with integration of *galU* and expression of *calS8* and *calS9.* Finally, the engineered host *E. coli* (US89Gt-4) (Fig. 2) was prepared and used to produce glycosylated flavonoids.

Engineered host *E. coli* (US89Gt-4) was grown under optimal conditions and the feeding experiment was carried out as described in Materials and Methods. Naringenin glycosylated with UDP-D-xylose was observed after extraction of the 60-h culture. The isolated product was analyzed by TLC (data not shown) and LC/MS analysis. Naringenin 7-*O*-xyloside was observed at retention time 8.92 min (Fig. 3). To determine whether the cells could make the glucosides in the resting state, *E. coli* (US89Gt-4) was transferred to phosphate buffer and cultured in the presence of 0.25% glycerol as an extra energy source. Aglycone was largely recovered from the cell pellet and we were unable to detect 7-*O*-xylosylated naringenin.

Naringenin 7-*O*-xyloside production was further confirmed by ESI-MS/MS analysis. MS-MS spectra of flavonoid glycosides had typical patterns, which depend mainly on the number or nature of the bound saccharides and their *O*-glycosidic linkages (Ferreres et al., 2004). Fragmentation of an *O*-glycoside starts from the cleavage of the *O*-sugar bond, and this behavior is useful for identifying the aglycone (Stobiecki, 2000). The loss of fragments with well defined mass from the pseudomolecular ion can provide precise information about the linked saccharide. Figure 3C presents the ESI-MS/MS spectrum in positive mode of an *O*-xylosylate-substituted flavonone, *i.e.*, naringenin 7-*O*-xyloside. The m/z 405 is the aglycone naringenin fragment of naringenin 7-*O*-xyloside. This fragment is concomitant with the expected pathway, *i.e.*, the loss of the xylose moiety. On the other hand, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-H_2O-CO]^+$, $[M+H-H_2O-2CO]^+$ ions were also observed (Boue et al., 2003; Chen et al., 2003; Ma et al., 1997).

CONCLUSION

Glycosylation is required for the biological activities of some flavonoids because the sugar usually participates in the molecular recognition of their cellular targets (He and Liu, 2002). Naringenin has been evaluated in the chemoprevention of carcinogenesis, inhibition of human cancer cell proliferation and delay of tumorigenesis (So et al., 1996; Tanaka, 1997). Because of the high importance of naringenin, it was selected as a substrate for production of a glycosylated product. In this study, *E. coli* was metabolically engineered to produce spatially distributed naringenin glycosidase *in vivo*. For the biosynthesis of UDP-D-xylose, three genes *galU*, *calS8* and *calS9* were integrated and expressed in *E. coli*; the *arGt-4* glycosyltransferase gene was also expressed to produce the final engineered host

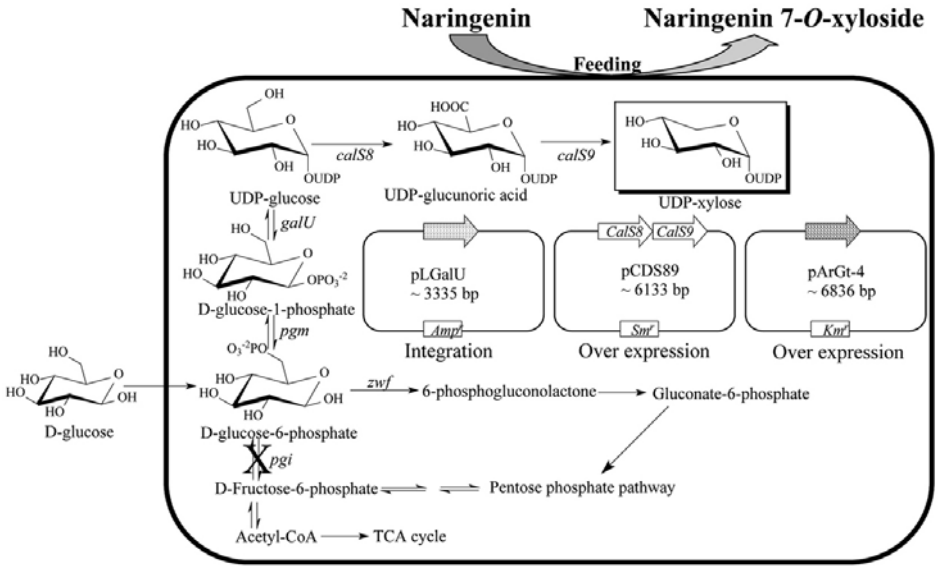

Fig. 2. *E. coli* engineered host *E. coli* (US89Gt-4) for production of glycosylated flavonoids

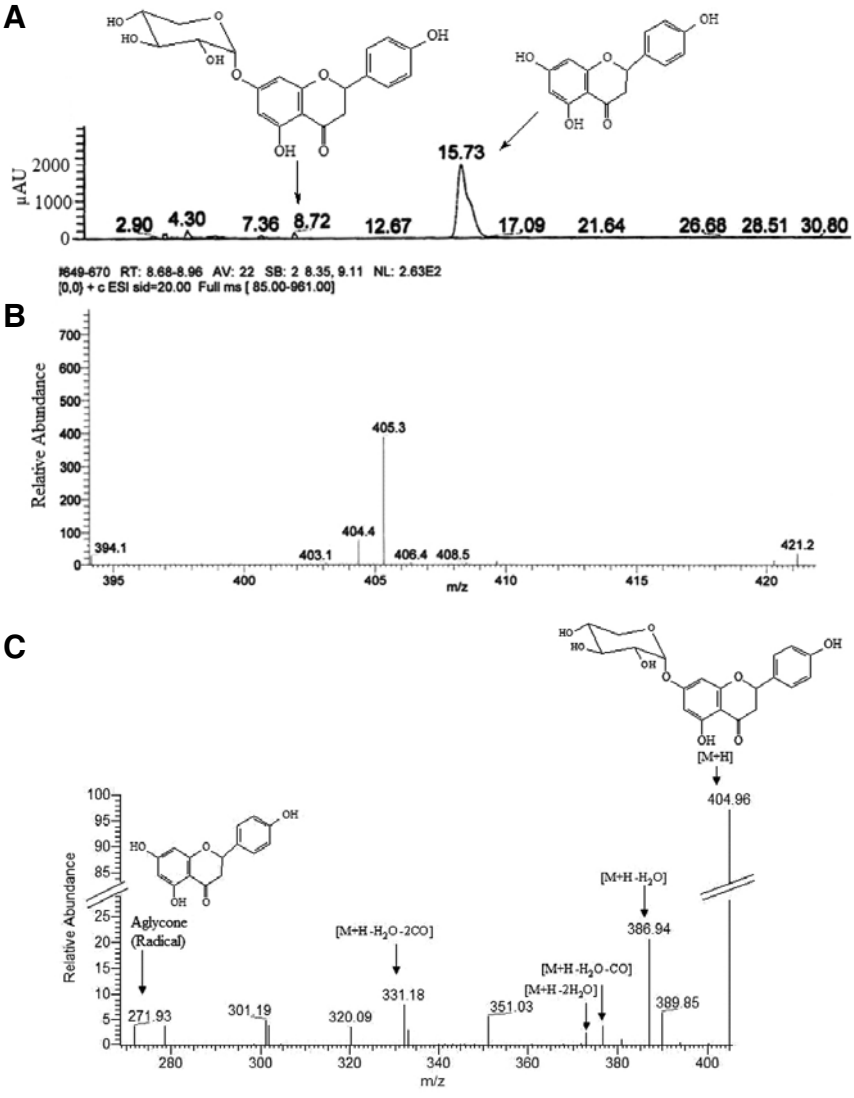

Fig. 3. (A) HPLC chromatogram of the product isolated from engineered host. The 7-*O*-xylosylated naringenin was detected at 8.72 min and naringenin at 15.73 min. (B) LC-MS chromatogram of 7-*O*-xylosylated naringenin (m/z^+ 405) in positive mode (C) ESI/MS-MS spectrum in positive mode of 7-*O*-xylosylated naringenin.

E. coli (US89Gt-4). To validate the engineered host, the bacteria was fed with 0.2 mM naringenin with glycerol (0.25%) as an additional carbon source. The product was isolated and analyzed by HPLC, LC/MS and ESIMS/MS and confirmed as naringenin 7-*O*-xyloside. We also attempted to optimize the production yield by changing culture media or IPTG concentrations but we couldn't increase the production level of 7-*O*-xylosyl naringenin to date might be because of low expression level of the enzymes (*calS8* and *calS9*) for UDP sugars pool which is under study. As well as other experimental procedures to increase the production level of naringenin 7-*O*-xyloside are currently under investigation in our laboratory.

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