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RESEARCH PAPER

Exploration of Glycosylated Flavonoids from Metabolically Engineered *E. coli*

Dinesh Simkhada, Nagendra Prasad Kurumbang, Hei Chan Lee, and Jae Kyung Sohng

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Abstract Flavonoids glycosylated with UDP-glucuronic acid and UDP-xylose are spatially distributed in nature. To produce these glycosides, *E. coli* was engineered to overexpress biosynthetic gene clusters of UDP-sugars (*galU* from *E. coli* K12, UDP-glucose dehydrogenase (*calS8*), and UDP-glucuronic acid decarboxylase (*calS9*) from *Micromonospora echinospora* spp. *calichensis*). Flavonoids were glycosylated by overexpression of the glycosyltransferase gene (*atGt-5*) from *Arabidopsis thaliana*. Finally, metabolically engineered host *E. coli* (US89Gt-5) was generated. Production of flavonoid glycosides was observed in a biotransformation system consisting of flavonoids (naringenin and quercetin) exogenously fed to host cells. The glycosylated derivatives 7-O-glucuronyl naringenin (m/z^+ 449), 7-O-xylosyl naringenin (m/z^+ 405), and 7-O-glucuronyl quercetin (m/z^+ 479) were detected and confirmed by ESI-MS/MS, ESI-MS/MS and LC/MS-MS analysis, respectively.

Keywords: *E. coli* (US89Gt-5), metabolic engineering, UDP-sugars, 7-*O*-glucuronyl naringenin, 7-*O*-xylosyl naringenin, 7-*O*-glucuronyl quercetin *etc*.

1. Introduction

Flavonoids are natural compounds with diverse biological functions that are commonly used as pigments or cosmetic additives. Flavonoids possess immunodeficiency, antioxidant, and antimicrobial activities [1], whereas in plants they are known as UV protectants, activators of nodulation, and anti-microbial agents. Use of flavonoids as pharmaceutical agents has been hindered due to their low water solubility, a problem which can overcome by modification with sugar molecules [2]. In addition, intensive research has focused on the creation of glycoside libraries of secondary metabolites using several enzymes obtained from microorganisms [3].

Glycosylation is important for the biological activities of several antibiotics as well as numerous anti-cancer, anti-parasitic, and anti-fungal agents [4,5]. Sugar residues added *via* glycosylation play crucial biological roles in many natural products, and thus their removal often results in loss of biological activity [5,6]. Altering and/or exchanging the structures of these sugars or the points of aglycone attachment in natural products can enhance physiological properties. In this manner, the deoxysugar biosynthetic pathways of a producing bacterial strain can be altered using gene disruption and/or heterologous gene expression methods in order to reroute sugar biosynthetic intermediates to produce new products [7,8]. Alternatively, new biosynthetic pathways can be activated in hosts that do not normally produce glycosylated natural products. Furthermore, such genetically-engineered bacteria can be fed non-native aglycone or transformed with additional plasmids to produce novel compounds in combinatorial fashion [9].

Family 1 glycosyltransferase (UGTs) enzymes are characterized by utilization of UDP-sugar moieties as donor molecules, and they contain a conserved UGT-defining sequence motif near their C-termini [10,11]. This UGT-defining motif is often the only significant region of similarity regarding sequence alignments within and across phyla. Glycosylation reactions serve to convert reactive and toxic aglycones into more stable and non-reactive forms of storage. In

Dinesh Simkhada, Nagendra Prasad Kurumbang, Hei Chan Lee, and Jae Kyung Sohng*
Institute of Biomolecule Reconstruction (iBR), Department of Pharmaceutical engineering, Sun Moon University, Chungnam 336-708, Korea
Tel: +82-41-530-2246; Fax: +82-41-544-2919
E-mail: sohng@sunmoon.ac.kr

addition, attachment of a hydrophilic glucose moiety to hydrophobic aglycones increases water solubility. Whereas UDP-glucose and UDP-glucuronic acid are considered the most typical donor molecules for family 1 glycosyltransferases, examples of UDP-rhamnose, UDP-xylose, and UDP-galactose also exist [12]. UDP-glucose is the starting point of the synthesis of other UDP-sugars (such as UDP-glucuronic acid and UDP-xylose) [13]. The synthesis of UDP-glucuronic acid (UDP-GlcA) can be obtained by oxidation of UDP-glucose, which is catalyzed by NAD-dependent UDP-glucose dehydrogenase and synthesis of UDP-xylose (UDP-Xyl) obtained from decarboxylation of UDP-GlcA [13].

Production of glycosylated flavonoids from metabolically-engineered *E. coli* is very challenging due to the unavailability of UDP-sugars. To circumvent this bottleneck, *E. coli* was metabolically engineered with the UDP-sugar pathway for synthesis of UDP-sugars and the glycosyltransferase gene from *Arabidopsis thaliana*, resulting in glycosylation of the substrate. As non-native aglycones, naringenin and quercetin were used as starting materials for biosynthesis of more complicated flavonoids with high biological impact. The metabolically-engineered system of *E. coli* was expected to produce 7-*O*-glucunoryl as well as 7-*O*-xylosyl flavonoids.

2. Materials and Methods

2.1. Strains, plasmids, and chemicals

Escherichia coli XL1-Blue (MRF) (Stratagene, USA) was used as a host cell for the preparation of recombinant plasmids and manipulation of DNA, whereas *E. coli* BL21 (DE3) Δ*pgi* deletion mutant was used for the biotransformation of recombinant. pGEM-T easy vector (Promega, USA), pLOI2223 (Integration vector, National Bioresource Project NIG, Japan), pET28a+, and CDFDuet-1(Novagen, Germany) were used as vectors for the cloning of the polymerase chain reaction (PCR) products, integration, and gene expression. The strains and plasmids used are summarized in detail in Table 1. All chemicals used in this study were purchased from Sigma (St. Louis, USA). Naringenin, quercetin, isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin, kanamycin, and spectinomycin were also used in the experiment. All restriction enzymes used for the cloning process were obtained from Takara (Japan). Other chemicals were of the highest grade commercially available.

2.2. DNA manipulation

DNA preparation, manipulation, restriction endonuclease digestion, and ligation were carried out according to standard protocols. cDNA of the *Arabidopsis thaliana* glycosyltransferase (**AF360262**) gene was purchased from RIKEN Bioresource Centre (http://www.brc.riken.jp/inf/en/index.shtml). After DNA manipulation, the absence of undesired alterations during PCR was verified by nucleotide sequencing using an automated nucleotide sequencer.

2.3. Construction of pLOIGalU, pCDS89, and pAtGt-5

Recombinants were constructed for *E. coli*. The primers, restriction sites, and cloned vector are summarized in detail in Table 2. PCR products were cloned in pGEM-T easy vector and sequenced prior to cloning into the expression

Table 1. Bacterial strains, plasmids, and cDNA used in this study

Strains and plasmids	Relevant characterstics	Source or references
Strains		
E. coli XL1 blue	General cloning host	Stratagene PBL company
E. coli Bl21DE3	Expression host	Stratagene PBL company
E. coli Bl21DE3 (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
pCDS89	pCDFDuet-1 containing calS8 and *calS9*	This work
pLOIGalU	Integration vector pLOI2223 containing *galU*	This work
pAtGt-5	Expression vector pET28a+ containing *argt-2*	This work
cDNA	**NCBI Acession Number**	**Source**
AtGt-5	AF360262	RIKEN Bioresource center

Table 2. PCR primers used in amplification of genes and integration plasmids

Gene	Sequence (5'-3')	Portion of gene	Restriction site	Vectors
S8D	AGCGGATCCCATCATGCCGTTCCTTCC	5'	*BamH*I	CDFDuet-1
	AGCAAGCTTTCACCTTCCAATGCCGC	3'	*Hind*III	
S9D	AACCATATGCCCAGATCCCTGGTCACC	5'	*Nde*I	CDFDuet-1
	AGTCTCGAGCTACCTGACGACCAGTCC	3'	*Xho*I	
GalU	AGCGAATTCATGGCTGCCATTAATACG	5'	*EcoR*I	pLOI2223
	GCAGGATCCTTACTTCTTAATGCCCAT	3'	*BamH*I	
AtGt-5	AGCGGATCCATGGAGGAATCCAAAACA	5'	*BamH*I	pET28a+
	GACAAGCTTTTAGTGGTTGCCATTTTG	3'	*Hind*III	

vector. PCR was performed using a thermocylcer (Takara, Japan) under the following conditions: 30 cycles of 30 sec at 94°C, 1 min at 60°C, and 1 min at 72°C. The plasmid pLOIGalU was constructed by amplification of *galU* from *E. coli* K12, which was cloned into the integration vector pLOI223. Plasmid pCDS89 was constructed by amplification of *calS8* and *calS9* from the recombinant used in our previous study provided by Prof. Andreas Bethold that was cloned into pCDFDuet, and plasmid pAtGt-5 was constructed by amplification of *Arabidopsis thaliana* cDNA that was cloned into pET28a+.

2.4. Experimental design for detection of glycosylation

The glucose-phosphate isomerase (*pgi*) gene was disrupted from the *E. coli* BL21(DE3) chromosome using a λ red mediated Quick and Easy BAC Modification Kit (GENE BRIDGES) to generate the host *E. coli* BL21(DE3) Δpgi [14]. The recombinant of integration vector pLOIGalU was transferred into *E. coli* BL21 (DE3) Dpgi by heat pulse transformation. The antibiotic-resistant transformants were then selected and further conformed by isolation plasmid DNA, and this was verified by PCR using respective gene primers. In the same manner, recombinants pCDS89 and pAtGt-5 were further transformed in a *gal*U-integrated host. Finally, engineered *E. coli* (US89Gt-5) was constructed for the production of glycosylated products.

2.5. Whole cell biotransformation assay

E. coli (US89Gt-5) was used for whole cell biotransformation assay. This strain was grown on LB medium. After the bacterial culture had reached an OD_{600} of 0.7, 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added, followed by 0.2 mM Naringenin and Quercetin separately after 30 min and then 0.25% glycerol as a carbon source. After 60 h, the cultural broth was harvested for compound extraction. Supernatant was extracted using ethyl acetate and cell pellets were extracted using 80% methanol. The extracted compounds were concentrated and dissolved in methanol. The compounds recorded in this process were analyzed by ESI-MS/MS and LC-MS/MS.

2.6. ESI, ESI-MS/MS, and LC-MS/MS Analysis

Electron spray ionization-mass spectrometry (ESI-MS) analysis was carried out at a probe temperature of 392°C with a source voltage of 31.2 V using a Finnigan AQA (UK) mass spectrometer. Samples (2 μL) were injected *via* a 10 μL loop and then transferred at a flow rate 1 mL/min using methanol as a solvent. LC-MS analysis were performed with a C18 column (30105-254630, Thermo, ODS Hypersil; 4.6 × 250 mm; 5 μm diameter particle) using an isocratic gradient consisting of 70% water (0.1% trifluroacetic acid) and 30% acetonitrile at a flow rate of 1 mL/min. The absorbance was recorded at 290 and 330 nm.

3. Results and Discussion

3.1. Engineering of UDP-sugars pathway

Metabolic engineering has become an effective tool for the development of new antibiotics, and it may provide important strategies in the fight against antibiotic-resistant pathogens [15,16]. The goal of our research was to construct an *E. coli* strain capable of synthesizing UDP-sugars, which would then be used to glycosylate flavonoids. As the synthesis of UDP-glucose is a prerequisite to the production of UDP-sugars, maximum carbon flux into UDP-glucose seems to be a logical first step of metabolic engineering [13]. Phosphoglucose isomerase (pgi) increases the flux from glucose-6-phosphate to fructose-6-phosphate, which indicates that deletion of *pgi* would be important to partitioning the flux toward glucose-1-phosphate from glucose-6-phosphate. Therefore, *E. coli* BL21 (DE3)/pgi [14], was used as a host for further engineering of the desired product. UDP-glucose pyrophosphorylase (GalU), an enzyme involved in UDP-glucose synthesis, was overexpressed in order to increase UDP-glucose synthesis. To increase the flux towards UDP glucose, GalU was introduced into the integration vector pLOI2223 under the assumption that accumulation of UDP-glucose was sufficient to direct the pathway. Further, recombinant containing CalS8 and CalS9 (pCalS89) (Fig. 1), which direct the

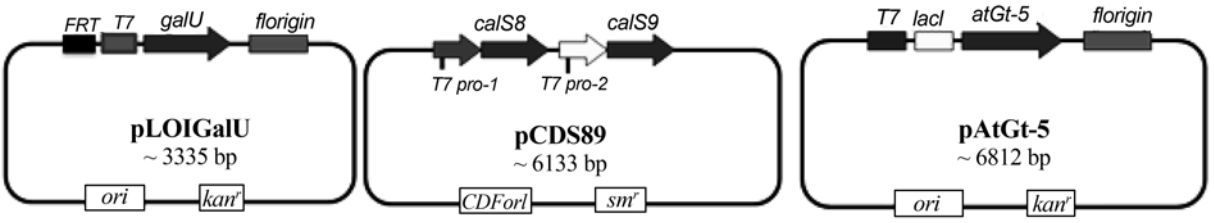

Fig. 1. Recombinants constructed for engineering of metabolic pathways in *E. coli*: (A) Recombinant plasmid of the *galU* gene from *E. coli* K12 in integration vector pLOI2223, (B) *calS8* and *calS9* genes were cloned into CDFDuet-1 vector, and (C) 7-*O*-glycosyltransferase cloned into pET28a+ expression vector for construction of pAtGt-5.

pathway towards UDP-glucuronic acid and UDP-xylose, was inserted into the GalU-integrated host for overexpression. The engineered host expressing these genes was able to synthesize UDP-sugars for the glycosylation of flavonoids upon expression of recombinant pAtGt-5.

3.2. Gene sequence analysis of AtGt-5

AtGt-5 (accession number AF360262) from *Arabidopsis thaliana* used in this study belongs to the GT-B superfamily. This family includes Leloir pathway enzymes, including that which attaches UDP-galactose to ceramide during the biosynthesis of galactosylceramide [17]. AtGt-5 contained an amino acid sequence present in several plant secondary product glycosyltransferase (PSPG box). The protein sequence of AtGt-5 was compared to that of 7-O-glycosyltransferase from several strains (Fig. S1). It was found that the entire conserved region of AtGt-5 resembled that of 7-O-glycosyltransferase from several strains, which further assisted the prediction of glycosylation position. The complete ORF (1,443 bp) of *atGt-5* was cloned into expression vector pET28a+ and then transferred into *E. coli* BL21 (DE3) in order to verify the expression level. The ~60 kDa protein resembled the expected size of the recombinant (Fig. S2).

3.3. Feeding experiments

The main goal for the construction of a metabolically-engineered strain was to obtain an improved system for

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                *        20         *        40         *        60         *        80         *
Uf7gt    : MG------QLHIVLVPMIAHGHMIPMLDMAKLFSSRGVKTTIIATPA-----FAEPIRKARESG---HDIGLTTTKFPPKGSSLPDNIR----- :  75
At_2     : MGTPVEVSKLHFLLFPFMAHGHMIPTLDMAKLFATKGAKSTILTTPLNAKLFFEKPIKSFNQDNPGLEDITIQILNFPCTELGLPDGCENTDFI :  94
ArGt-2   : ---MEESKTPHVAIIPSPGMGHLIPLVEFAKRLVHLHG---LTVTFVIAGEGPPSKAQRTVLDS-------LPSSISSVFLPPVDLTDLSSSTR :  81
GmIF7Gt  : -------MKDTIVLYPNLGRGHLVSMVELGKLILTHHPSLSITILILTPPTTPSTTTTTLACDSNAQYIATVTATTPSITFHRVPLAALPFNTP :  87

           100        *       120        *       140        *       160        *       180
Uf7gt    : --SLDQVTDDLLPHFFRALELLQEPVEEIMEDLKPDCLVSDMFLPWTTDSAAKFGIPRLLFHGTSLFARCFAEQMSIQKPYKNVSSDSEPFVLR : 167
At_2     : FSTPDLNVGDLSQKFLLAMKYFEEPLEELLVTMRPDCLVGNMFFPWSTKVAEKFGVPRLVFHGTGYFSLCASHCIRLPK---NVATSSEPFVIP : 185
ArGt-2   : IES--RISLTVTRSNPELRKVFDSFVEGGRLPTALVVDLFGTD---AFDVAVEFHVPPYIFYPTTANVLSFFLHLPKLDETVS-CEFRELTEPL : 169
GmIF7Gt  : FLPPHLLSLELTRHSTQNIAVALQTLAKASNLKAIVIDFMNFNDPKALTENLNNNVPTYFYYTSGASTLALLLYYPTIHPTLIEKKDTDQPLQI : 181

          *       200        *       220        *       240        *       260        *       280
Uf7gt    : GLPHEVSFVRTQIPDYELQEGGDDAFSKMAKQMRDADKKSYGDVINSFEELESEYADYNKN--VFGKKAWHIGPLKLFNNRAEQKSSQRGKESA : 259
At_2     : DLPGDILITEEQVMETEE----ESVMGRFMKAIRDSERDSFGVLVNSFYELEQAYSDYFKS--FVAKRAWHIGPLSLGNRKFEEKAE-RGKKAS : 272
ArGt-2   : MLPGCVPVAGKDFLDPAQDR--KDDAYKWLLHNTKRYKEAEGILVNTFFELEPNAIKALQEPGLDKPPVYPVGPLVN---------IGKQEAKQ : 252
GmIF7Gt  : QIPGLSTITADDFPNECKDP--LSYACQVFLQIAETMMGGAGIIVNTFEAIEEEAIRALSEDATVPPPLFCVGPVIS---------APYG---- : 260

                 *       300        *       320        *       340        *       360        *
Uf7gt    : IDDHECLAWLNSKKPNSVVYMCFGSMATFTPAQLHETAVGLESSGQDFIWVVR----------NGG---ENEDWLPQGFEERIKGKGLMIRGW : 339
At_2     : IDEHECLKWLDSKKCDSVIYMAFGTMSSFKNEQLIEIAAGLDMSGHDFVWVVN----------RKGSQVEKEDWLPEGFEEKTKGKGLIIRGW : 355
ArGt-2   : TEESECLKWLDNQPLGSVLYVSFGSGGTLTCEQLNELALGLADSEQRFLWVIRSPSGIANSSYFDSHSQTDPLTFLPPGFLERTKKRGFVIPFW : 346
GmIF7Gt  : EEDKGCLSWLNLQPSQSVVLLCFGSMGRFSRAQLKEIAIGLEKSEQRFLWVVRTELGGAD----DSAEELSLDELLPEGFLERTKEKGMVVRDW : 350

          380        *       400        *       420        *       440        *       460        *
Uf7gt    : APQVMILDHPSTGAFVTHCGWNSTLEGICAGLPMVTWPVFAEQFYNEKLVTEVLKTGVSVGNKK-WQRVGEGVGSEAVKEAVERVMVGDGAAEM : 432
At_2     : APQVLILEHKAIGGFLTHCGWNSLLEGVAAGLPMVTWPVGAEQFYNEKLVTQVLKTGVSVGVKKMMQVVGDFISREKVEGAVREVMVGE---ER : 446
ArGt-2   : APQAQVLAHPSTGGFLTHCGWNSTLESVVSGIPLIAWPLYAEQKMNAVLLSEDIRAALRPRAGD-----DGLVRREEVARVVKGLMEGEEGKGV : 435
GmIF7Gt  : APQAAILSHDSVGGFVTHCGWNSVLEAVCEGVPMVAWPLYAEQKMNRMVMVKEMKVALAVNENK-----DGFVSSTELGDRVRELMESDKGKEI : 439

                 480        *       500        *
Uf7gt    : RSRALYYKEMARKAVEEGGSSYNNLNALIEELSAYVPPMKQGLN- : 476
At_2     : RKRAKELAEMAKNAVKEGGSSDLEVDRLMEELT-LVKLQKEKV-- : 488
ArGt-2   : RNKMKELKEAACRVLKDDGTSTKALSLVALKWKAHKKELEQNGNH : 480
GmIF7Gt  : RQRIFKMKMSAAEAMAEGGTSRASLDKLAKLWKQS---------- : 474
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Fig. S1. Gene sequence analysis of AtGt-5 (Arabidopsis thaliana) expressing Uf7gt (*Scutellaria baicalensis*, Gene Bank accession no. BAA83484); At_2 (*Arabidopsis thaliana*, Gene Bank accession no. AY090273); GmIF7GT (*Glycine max*, Gene Bank accession no. AB292164). The entire specific conserved regions for plant glycosyltransferase are underlined.

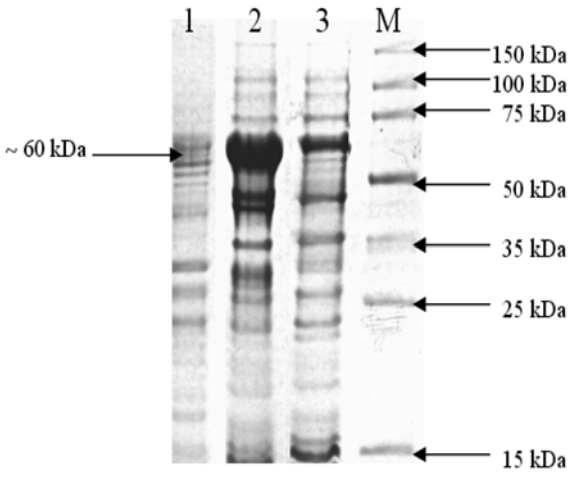

Fig. S2. SDS–PAGE analysis of AtGt-5, Lane 1: protein without induction, Lane 2: Total expressed protein, and Lane 3: Soluble protein, M: Marker.

flavonoid glycosylation. The engineered *E. coli* (US89Gt-5) strain was constructed by integration of pLOIGalU, and expression of pCDS89 and pAtGt-5 were used for the bioconversion experiments (Fig. 2). Cultural broth of the strain was induced with 0.2 mM IPTG and was fed with naringenin and quercetin separately to a final concentration of 0.2 mM in different flasks. Culture was continued for 60 h at 20ºC, after which the products were extracted and analyzed. Flavonoids glycosylated with UDP-xylose were not detected during our experiment perhaps due to insufficient levels of UDP-xylose synthesized for glycosylation. As a control experiment, we performed the experiment without expression of pAtGt-5 and detected no glycosylated products.

3.4. Analysis of glycosylated compounds

The extracted compounds were first analyzed by ESI/MS analysis. The mass profiles of the isolates fed with naringenin revealed the masses of glucunoryl naringenin (m/z^+ 449), xylosyl naringenin, and glucunoryl quercetin (m/z^+ 479) (data not shown). Further analyses were carried out by ESI-MS/MS and LC-MS/MS. Fragmentation of *O*-glycoside was initiated by cleavage of the *O*-sugar bond, and this behavior was useful for identifying the aglycone [18]. The loss of fragments with well-defined masses from the pseudo molecular ion provided precise information about the linked saccharide. Similarly, Fig. 4A presents the positive ESI-MS/MS spectrum of glucunoryl naringenin with a parent mass of m/z^+ 449. The fragment of m/z^+ 449 was concomitant with the expected pathway, *i.e.*, the loss of glucunoride moiety (m/z^+ 273, naringenin). The parent ion also contained the fragments $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ as demonstrated previously by several researchers [19-21]. Similarly, Fig. 4B represents the ESI-MS/MS spectrum of xylosyl naringenin with a parent mass of m/z^+ 405. The fragmentation pattern resembled in a similar pattern as mentioned earlier, *i.e.*, the loss of xylosyl moiety (m/z^+ 273, naringenin). The LC-MS/MS spectrum of 7-*O*-glucuronyl quercetin (Fig. 5) provided the fragment of aglycone quercetin (m/z^+ 303) by the loss of glucunoride moiety (m/z 176). These detailed spectrums from the mass

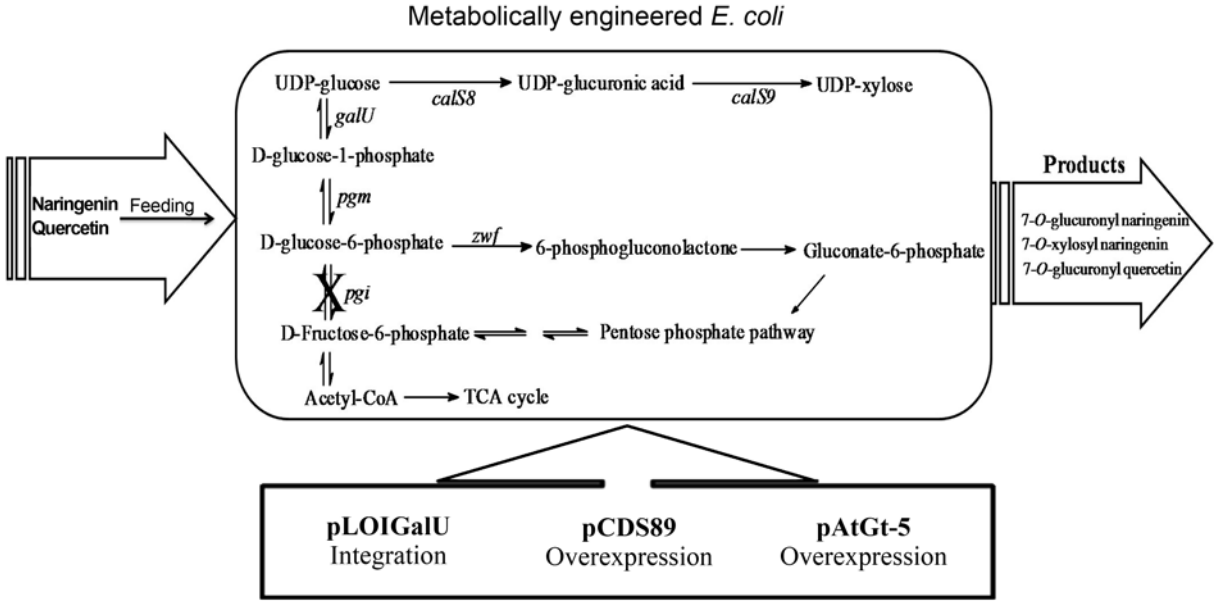

Fig. 2. Metabolically-engineered host *E. coli* (US89Gt-5) for production of spatially-distributed glycosylated flavonoids.

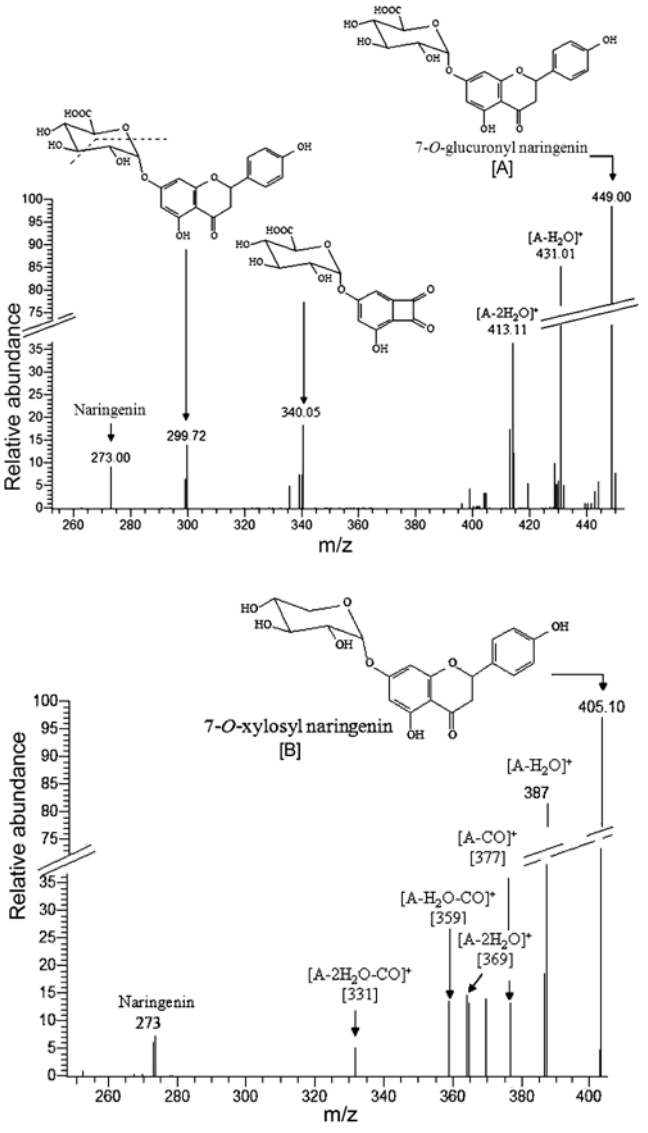

Fig. 3. ESI-MS/MS analysis spectrum in positive mode. (A) 7-*O*-glucuronyl naringenin and (B) 7-*O*-xylosyl naringenin.

to mass analysis suggest that the isolates from the host were glycosyalted flavonoids.

4. Conclusion

Metabolic engineering of *E. coli* was carried out to produce spatially-distributed glycosidase flavonoids *in vivo*. pLOIGalU and pCDS89 recombinants were applied to engineer UDP-sugars pathways, whereas pAtGt-5 was used for glycosylation. To confirm successful engineering, the host was fed 0.2 mM of each flavonoid, with glycerol (0.25%) as an additional carbon source. The products were isolated and analyzed by ESI-MS/MS and LC-MS/MS, and the detected glycosylated products were confirmed as

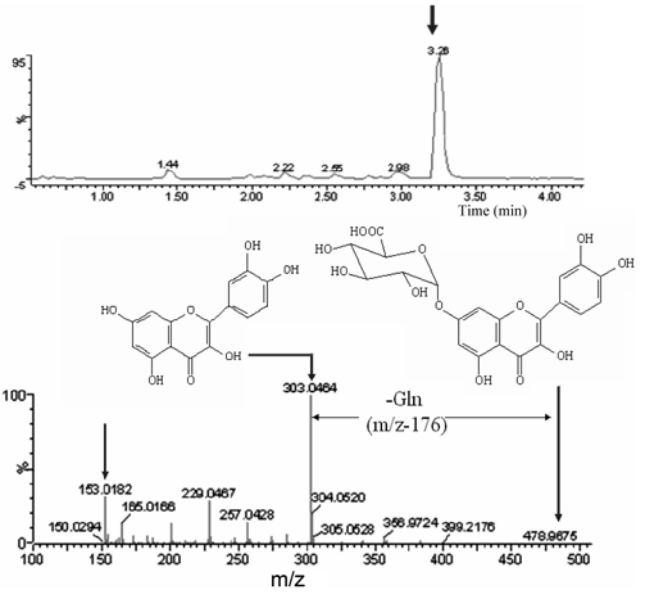

Fig. 4. LC-MS profile with LC-MS/MS analysis spectrum of 7-*O*-glucuronyl quercetin.

7-*O*-glucuroyl naringenin, 7-*O*-xylosyl naringenin, and 7-*O*-glucuronyl quercetin.

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