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# Production of Bioactive Flavonol Rhamnosides by Expression of Plant Genes in *Escherichia coli*

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**ABSTRACT:** Biotransformation of flavonoids using *Escherichia coli* harboring specific glycosyltransferases is an excellent method for the regioselective synthesis of flavonoid glycosides. Flavonol rhamnosides have been shown to contain better antiviral and antibacterial activities compared to flavonol aglycones. To synthesize flavonoid rhamnoside, a strain of *E. coli* expressing UDP-rhamnose flavonol glycosyltransferase (*AtUGT78D1*) from *Arabidopsis thaliana* was used to produce quercetin 3-*O*-rhamnoside. The biotransformation of quercetin using this *E. coli* transformant resulted in the production of quercetin 3-*O*-rhamnoside as a major product. A strain of *E. coli* rfbD (encoding dTDP-4-dehydrorhamnose reductase) expressing *AtUGT78D1*, which is involved in the final step of thymidine diphosphate rhamnose (TDP-rhamnose) biosynthesis, did not produce quercetin 3-*O*-rhamnoside, meaning that AtUGT78D1 used endogenous TDP-rhamnose as a sugar donor to produce quercetin 3-*O*-rhamnoside. The production of quercetin 3-*O*-rhamnoside could be increased by up to 160% by co-expressing *AtUGT78D1* and rhamnose synthase gene 2 (*RHM2*), which catalyzes the conversion of UDP-glucose into UDP-rhamnose. Using an *E. coli* strain harboring *AtUGT78D1* and *RHM2*, 150 mg/L quercetin 3-*O*-rhamnoside and 200 mg/L kaempferol 3-*O*-rhamnoside were produced in 48 h.

**KEYWORDS:** *Glycosyltransferase, rhamnose synthase, flavonoid-rhamnoside*

## INTRODUCTION

Phytochemicals, including flavonoids, are good sources of new medicine. Flavonoids exert various biological effects, including anticancer, antibacterial, antiviral, and immunostimulatory effects.[1] To date, more than 9000 flavonoids have been described,[2] some of which are conjugated with sugars, such as glucose, xylose, arabinose, glucuronic acid, and rhamnose.[3] Flavonol 3-*O*-rhamnosides, such as kaempferol 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside, have diverse biological activities. For example, kaempferol 3-*O*-rhamnoside inhibits the proliferation of breast cancer cells[4] and absorption of glucose in the intestines,[5] and quercetin 3-*O*-rhamnoside promotes skin whitening and ultraviolet protection by inhibiting tyrosinase, which is the first enzyme of the melanin biosynthesis.[6] Recently, pathogens, such as the influenza virus and antibiotic-resistant bacteria, have caused serious problems in human and animal epidemics, and in many such cases, there are no good treatments. As an alternative antimicrobial agent, quercetin 3-*O*-rhamnoside was shown to block replication of the influenza virus,[7,8] and kaempferol 3-*O*-rhamnoside has potent antibactial and antifungal activities.[9] Flavonol rhamnosides have been shown to contain better antiviral and antibacterial activities compared to flavonol aglycones. These flavonol 3-*O*-rhamnosides were originally isolated from plants, but extraction from plants has some drawbacks because of the limited supply of plant materials and the requirement for multiple purification steps. Chemical synthesis of flavonol 3-*O*-rhamnosides could be a solution to this problem, but regioselective synthesis might be a limiting factor. Therefore, biological synthesis may represent an alternative method to efficiently obtain large quantities of these compounds.

Flavonoid *O*-rhamnosides are synthesized by glycosyltransferase (GT) using thymidine diphosphate or uridine diphosphate rhamnose as the sugar donor and a flavonoid as the sugar acceptor.[11] The biosynthetic pathway of nucleotide rhamnoside is different in bacteria and plants. In *Escherichia coli* and other bacteria, thymidine diphosphate rhamnose (TDP-rhamnose) is synthesized from TDP-glucose by the action of three enzymes: dTDP-glucose 4,6-dehydratase (rfbB), dTDP-4-dehydrorhamnose 3,5-epimerase (rfbC), and dTDP-4-dehydrorhamnose reductase (rfbD) (Figure 1).[11] In plants, unlike in bacteria, a single multifunctional enzyme catalyzes the biosynthesis of uridine diphosphate rhamnose (UDP-rhamnose) from UDP-glucose. UDP-rhamnose serves as a sugar donor for the biosynthesis of rhamnose-containing polysaccharides in the primary cell wall and for secondary metabolite−rhamnose conjugates.[12,13] Thus far, two flavonoid GTs that use UDP-rhamnose as a sugar donor have been described.[14,15] AtUGT78D1 from *Arabidopsis thaliana* transfers rhamnose unit from UDP-rhamnose to the 3-hydroxy group of quercetin, while AtUGT89C1 uses quercetin 3-*O*-glucose as a sugar acceptor to synthesize quercetin 3-*O*-glucoside-7-*O*-rhamnoside. In the current study, AtUGT78D1 was used to synthesize quercetin 3-*O*-rhamnoside.

*E. coli* is a good model system for the synthesis of small molecules because it is easy to manipulate genetically, it can harbor a variety of vectors, and it has well-established metabolic pathways. Therefore, we used *E. coli* to synthesize flavonol 3-*O*-

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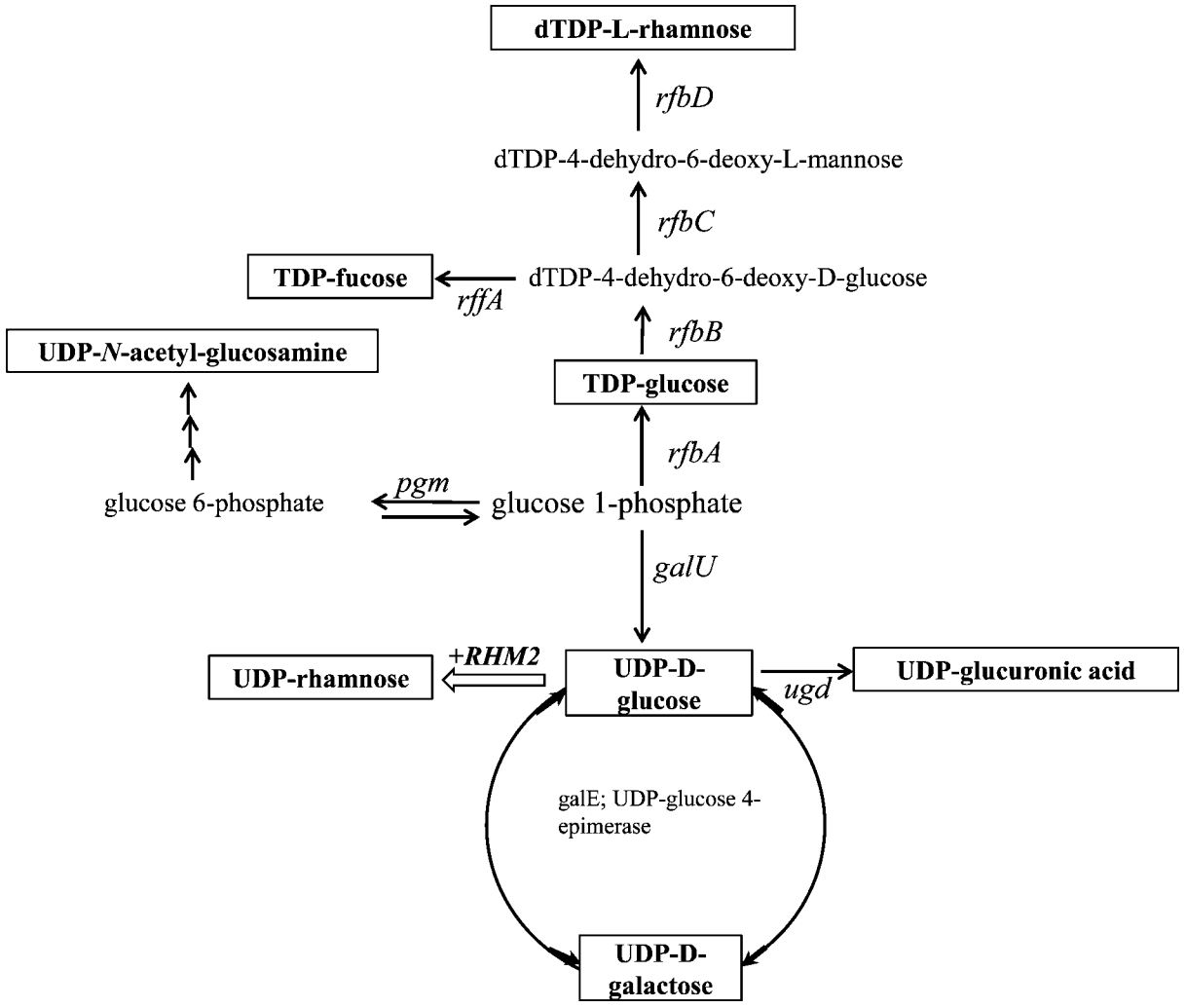


**Figure 1.** Nucleotide sugar biosynthesis pathway and production of UDP-rhamnose in *E. coli*. *galU*, UTP-glucose 1-phosphate uridyltransferase; *pgm*, phosphoglucomutase; *ugd*, UDP-glucose 6-dehydrogenase; *rffA*, dTDP-4-oxo-6-deoxy-D-glucose transaminase; *rfbA*, dTDP-glucose pyrophosphorylase; *rfbB*, dTDP-glucose 4,6-dehydratase; *rfbC*, dTDP-4-dehydrorhamnose 3,5-epimerase; *rfbD*, dTDP-4-dehydrorhamnose reductase; and *RHM2*, rhamnose synthase 2.

**Table 1. Details of the Plasmids and Strains Used in This Present Study**

| plasmids or *E. coli* strain | relevant properties or genetic marker | source or reference |
|---|---|---|
| | Plasmids | |
| pACYCDDuet | P15A ori, $Cm^r$ | Novagen |
| pCDFDuet | CloDE13 ori, $Str^r$ | Novagen |
| pGEX 5X-2 | pRR322 ori, $Amp^r$ | |
| pG-D1 | pGEX 5X-2 + *AtUGT78D2* from *A. thaliana* | this study |
| pC-RHM2 | pCDFDuet + *RHM2* from *A. thaliana* | this study |
| | Primers | |
| RHM2 forward | ATGGAATTCGATGGATGATACTACGTATAA | AF360160[13] |
| RHM2 reverse | CATGCGGCCGCTTAGGTTCTCTTGTTTGGT | |
| AtUGT78D1 forward | ATGAATTCATGACCAAATTCTCCGA | At1g53500[14] |
| AtUGT78D1 reverse | CATGCGGCCGCTAAACTTTCACAATTTCGT | |
| | Strains | |
| BL21 (DE3) | $F^-$ *ompT* $hsdS_B$ ($r_B^-$ $m_B^-$) *gal dcm lon* (DE3) | Novagen |
| BrfbD | BL21(DE3) Δ*rfbD* | Yoon et al.[26] |
| B201 | BL21(DE3) harboring pG-D1 | this study |
| B202 | BL21(DE3) harboring pG-D1 and pC-RHM2 | this study |
| B203 | BrfbD harboring pG-D1 | this study |
| B204 | BrfbD harboring pG-D1 and pC-RHM2 | this study |

rhamnoside. GT specific for flavonols (kaempferol and quercetin), and UDP (or TDP)-rhamnose were required because GTs normally have specificity for both the sugar donor and the sugar acceptor. A stable supply of the appropriate nucleotide conjugated to rhamnose is also required, because nucleotide-rhamnose is used not only for the production of flavonol 3-*O*-rhamnoside but also for cellular metabolism. With the expression of a flavonol glycosyltransferase, *AtUGT78D1* and *rhamnose synthase 2* (*RHM2*), both from *Arabidopsis thalia* in *E. coli*, flavonol 3-*O*-rhamnosides were successfully synthesized. This provides an efficient means of producing large quantities of flavonol 3-*O*-rhamnosides that could have applications as alternative antibiotics against viruses, bacteria, and fungi. In addition, this approach could be used to synthesize other rhamnoside derivatives, which might have novel biological activities.

## MATERIALS AND METHODS

**Plasmid Construction and *E. coli* Mutant Generation.** *AtUGT78D1* and *RHM2* were cloned by reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was isolated from 2-week-old plants of *A. thaliana* using the RNeasy Plant Mini Kit (Qiagen). Primers were designed on the basis of published nucleotide sequences of *AtUGT78D1* (GenBank accession number AF360160)[14] and *RHM2* (GenBank accession number At1g53500).[13] The primer sequences used to amplify *AtUGT78D1* and *RHM2* are given in Table 1. *AtUGT78D1* was subcloned into the *E. coli* expression vector pGEX 5X-2 (GE Healthcare Life Sciences), and *RHM2* was subcloned into pCDFDuet-1 (Novagen). The resulting constructs were named pG-D1 and pC-RHM2, respectively (Table 1).

The *rfbD* or *ugd* gene in *E. coli* BL21 (DE3) was deleted using the Quick and Easy *E. coli* Gene Deletion Kit (Gene Bridges, Heidelberg, Germany). The DNA-containing selection marker was amplified by PCR using the primers given in Table 1. *E. coli* BL21 (DE3) competent cells for electroporation were harvested and washed twice with ice-cold sterile 10% glycerol and resuspended in fresh sterile 10% glycerol. Electroporation was performed with a MicroPulser Electroporation Apparatus (BioRad). The deletion of *rfbD* was confirmed by PCR. The *E. coli* mutant strain with the *rfbD* deletion were named BrfbD (Table 1).

**Production of Flavonoid *O*-Rhamnoside in *E. coli*.** pG-D1 was transformed into the *E. coli* strains BL21 (DE3) and BrfbD. pC-RHM2 and pG-D1 constructs were co-transformed into *E. coli* strains BL21 (DE3), or BrfbD, and the resulting strains B202, and B204, were used to compare the production of quercetin 3-*O*-rhamnoside (Table 1). Enzymes were induced by adding 1 mM isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG) to the culture, and the culture was incubated at 25 °C for 18 h. Cells were harvested by centrifugation and resuspended to obtain a cell concentration corresponding to $OD_{600}$ of 3 in M9 medium containing 2% glucose. After quercetin (200 $\mu$M) was added to the cells, the reaction mixture was incubated at 30 °C for 4 h. The reaction mixture was extracted with ethylacetate. The supernatant was dried using vacuum centrifugation, dissolved in dimethyl sulfoxide (DMSO), and then analyzed by high-performance liquid chromatography (HPLC). The production of kaempferol 3-*O*-rhamnoside or quercetin 3-*O*-rhamnoside was calculated using kaempferol 3-*O*-glucoside or quercetin 3-*O*-glucoside as the standard, respectively, because neither of the former products are commercially available.

To determine the maximum production of flavonol 3-*O*-rhamnoside by strain B202, one-hundredth volume of the overnight culture was inoculated in a fresh Luria–Bertani (LB) medium and the culture was grown until the $OD_{600}$ reached 0.8. The enzymes were induced as described above. The cells were resuspended to obtain a cell concentration of $OD_{600}$ of 3 in 5 mL of M9 containing 2% glucose. Kaempferol (100 $\mu$M) and quercetin (100 $\mu$M) were added at 0, 3, 6, 12, and 24 h. The final concentration of kaempferol was 500 $\mu$M (143 mg/L), and that of quercetin was 400 $\mu$M (120.8 mg/L). The reaction mixture was incubated at 30 °C for 48 h. The reaction mixture (200 $\mu$L) was collected at 3, 6, 9, 12, 24, 36, and 48 h and centrifuged. The supernatant was used for HPLC analysis. Three independent experiments were performed.

Mass spectrometry (MS) was performed as described previously, with some modifications.[16] The LC–MS/MS instrument consisted of a Varian 212 HPLC (Varian, Palo Alto, CA), photodiode array detector (Varian 355), and a Varian 500-MS ion trap spectrometer. A Chromsep HPLC column (100 × 2.0 mm, 3 $\mu$m particle size, Varian) was used at a flow rate of 0.2 mL/min. The mobile phases consisted of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). Samples were run with the linear gradient from 15 to 30% B (v/v) in 15 min and to 100% B in 30 min and held at 100% B up to 35 min, after which it was decreased to 15%. The system was operated with MS workstation software (version 6.9.2, Varian, Inc.). Mass spectra were acquired using an electrospray ionization (ESI) source in negative ionization mode. The full-scan mass spectra were recorded for the range of $m/z$ 50–2000.

The structures of the reaction products were determined by nuclear magnetic resonance (NMR) spectroscopy.[17] For the large-scale purification, ion-exchange resin, instead of ethyl acetate extraction, was used. The reaction products were purified with an ion-exchange resin (Trilite Diaion, Sigma). A column (40 × 2.4 cm) was packed with resin, and after the reaction, the supernatant was applied to the column and washed with distilled water and the bound reaction products eluted with ethanol. The NMR data for quercetin 3-*O*-rhamnoside were as follows. $^1$H NMR (DMSO-$d_6$, 90 MHz) $\delta$: 7.66 (d, $J$ = 9.0 and 2.5 Hz, H-2′), 7.60 (d, $J$ = 2.5 Hz, H-6′), 6.84 (d, $J$ = 9.0 Hz, H-5′), 6.40 (d, $J$ = 2.5 Hz, H-8), 6.20 (d, $J$ = 2.5 Hz, H-6), 5.32 (d, $J$ = 8.0 Hz, H-1″), 4.34 (m, H-2″), 0.93 (s, H-6″). The NMR data for kaempferol-3-*O*-rhamnoside were as follows. $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.75 (d, $J$ = 8.8 Hz, H-2′ and H-6′), 6.91 (d, $J$ = 8.8 Hz, H-3′ and H-5′), 6.41 (d, $J$ = 2.0 Hz, H-8), 6.21 (d, $J$ = 2.0 Hz, H-6), 5.29 (d, $J$ = 1.3 Hz, H-1″), 0.79 (d, $J$ = 5.8 Hz, H-6″). It was compared to the previously published data.[18]

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

## RESULTS AND DISCUSSION

**Production of Quercetin 3-*O*-Rhamnose in *E. coli*.** In past reports of the biosynthesis of flavonoid glycosides using *E. coli* or yeast harboring GTs,[19−21] it was observed that flavonoid *O*-glucoside was a major reaction product. This is because most GTs previously used have a preference for UDP-glucose or TDP-glucose as the sugar donor. AtUGT78D1 is known to be a quercetin 3-*O*-rhamnoside transferase.[14] When expressed in *E. coli*, the resulting strain could transform exogenous quercetin into two products with HPLC retention times of 9.5 min (P1 in Figure 2B) and 11 min (P2 in Figure 2B). The molecular mass of P1 was 464 Da (Figure 2E), which indicated that it likely contained a glucose molecule, and the molecular mass of P2 was 448 Da (Figure 2F), which indicated the presence of a rhamnose molecule attached to quercetin. The structure of P2 was determined by NMR to be quercetin 3-*O*-rhamnoside. This result indicated that AtUGT78D1 used endogenous TDP-rhamnose as a rhamnose donor to produce quercetin 3-*O*-rhamnoside. According to EcoCyc,[22] TDP-rhamnose but not UDP-rhamnose is present in *E. coli* and is used for *O*-antigen biosynthesis.[23]

The ratio of quercetin 3-*O*-rhamnose/quercetin 3-*O*-glucose was approximately 2:1. This result is in contrast to that of a previous study, in which quercetin 3-*O*-glucoside but not quercetin 3-*O*-rhamnoside was produced in *E. coli* expressing *AtUGT78D1*.[24] To resolve this contradiction and confirm that AtUGT78D1 uses endogenous TDP-rhamnose to produce

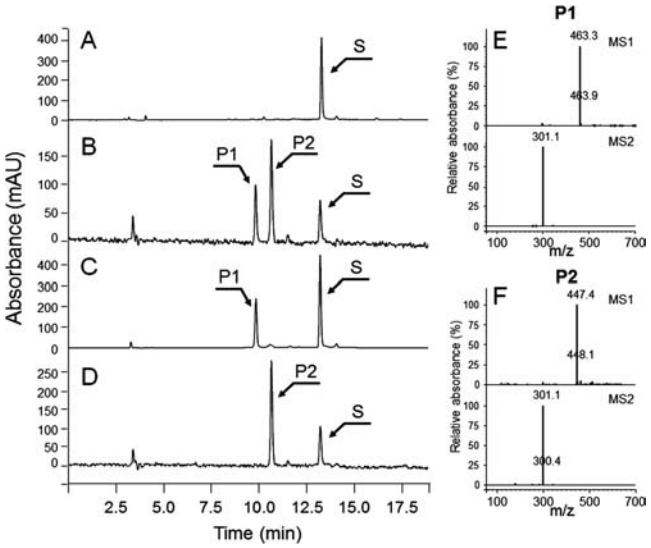


**Figure 2.** Analysis of the reaction products by HPLC. Quercetin was used as a substrate for biotransformation. (A) Authentic quercetin (S), (B) biotransformation of quercetin using wild-type BL21 strain harboring pG-D1, (C) biotransformation of quercetin using BrfbD strain harboring pG-D1, and (D) biotransformation of quercetin using BrfbD harboring pG-D1 and pC-RHM2. (E) MS/MS of reaction product 1 (P1) and (F) MS/MS of the reaction product (P2). S, quercetin; P1, quercetin 3-*O*-glucoside; and P2, quercetin 3-*O*-rhamnoside. The mass spectrometer (MS) was operated in the negative mode.

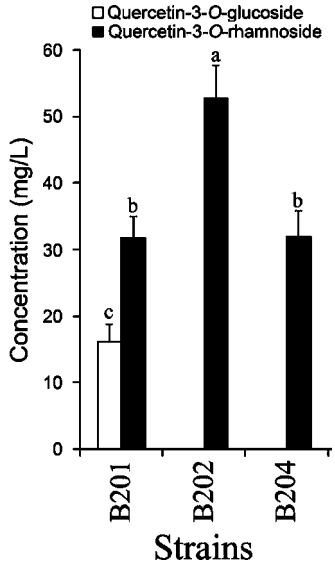


**Figure 3.** Comparison of quercetin 3-*O*-rhamnoside production in different *E. coli* strains. Bars with different letters indicate a significant difference at $p < 0.01$.

quercetin 3-*O*-rhamnoside, we deleted *rfbD*, which is required in the final step of TDP-rhamnose biosynthesis from TDP-glucose. The resulting mutant, BrfbD, was transformed with pG-D1, and biotransformation of quercetin was carried out. In the HPLC analysis of the reaction products, the peak corresponding to quercetin 3-*O*-rhamnoside disappeared (Figure 2C), indicating that endogenous TDP-rhamnose served as a sugar donor, but the mutant strain still produced quercetin 3-*O*-glucoside. That is, AtUGT78D1 used UDP-glucose when TDP-rhamnose was not available. Because the incubation time required for biotransformation was much longer than the time taken for the enzymatic reaction, even weak affinity of AtUGT78D1 for UDP-glucose could lead to the synthesis of quercetin 3-*O*-glucoside. This same effect was observed using AtUGT78D2.[25] Although the catalytic efficiency of AtUGT78D2 for UDP-*N*-acetylglucosamine was much lower than that for UDP-glucose, it used UDP-*N*-acetyglucosamine when UDP-glucose was not available.

**Overexpression of the *RHM2* Gene To Increase the Production of Quercetin 3-*O*-Rhamnoside.** *A. thaliana RHM2* catalyzed conversion of UDP-glucose into UDP-rhamnose.[10] RHM2 along with AtUGT78D1 was co-transformed into the *E. coli* strain BrfbD, and the resulting strain was named B204 (Table 1). We examined the production of quercetin 3-*O*-rhmanoside using strain B204. This strain converted quercetin to quercetin 3-*O*-rhamnoside with efficiency similar to that of strain B201, the wild-type strain (Figure 3). However, only a negligible amount of quercetin 3-*O*-glucoside was observed in B204 (Figure 2D), which suggested that the pool of UDP-glucose and dTDP-glucose decreased because RHM2 converted both nucleotide sugars to their corresponding nucleotide-rhamnoses. *In vitro* analysis showed that RHM2 uses dTDP-glucose as effectively as it uses UDP-rhamnose, although other rhamnose synthases, such as RHM1, uses UDP-glucose more effectively.[24] Taken together, these results indicated that the *RHM2* gene from *A. thaliana* complemented the rfbD mutant, which produced quercetin 3-*O*-rhamnose. While strain B201 produced only dTDP-rhamnose and strain B204 produced only UDP-rhamnose, both of these strains produced approximately the same amount of querecetin 3-*O*-rhamnoside. The relative yields of quercetin 3-*O*-rhamnoside in strains B201 and B204 seem to indicate that AtUGT78D1 uses dTDP-rhamnose as efficiently as it uses UDP-rhamnose. The production of dTDP-rhamnose or UDP-rhamnose in *E. coli* was not monitored because it is not easy to quantify nucleotide sugars in *E. coli*, and the production of quercetin-*O*-rhamnoside is directly proportional to the production of dTDP- and UDP-rhamnose.

Supplementation with UDP (or dTDP)-rhamnose is critical for the production of quercetin 3-*O*-rhamnoside in *E. coli*. To increase the amount of sugar donor (UDP-rhamnose), the *RHM* gene was overexpressed in *E. coli* BL21 (DE3). Strain B202, which overexpressed both *RHM2* and *AtUGT78D1*, was used to test the production of quercetin 3-*O*-rhamnoside. As shown in Figure 3, strain B202 produced approximately 55 mg/L quercetin 3-*O*-rhamnose, which is approximately 60% more than that produced by B201 or B204 (32 mg/L each). This strongly suggested that the pool of nucleotide-rhamnose was increased by *RHM2* expression, because of the fact that strain B202 has a biosynthetic pathway for dTDP-rhamnose (because *rfbD* is functional) and also for UDP-rhamnose (because of *RHM2* expression). However, strains B201 and B204 each have only one pathway: strain B201 has the dTDP-rhamnose biosynthetic pathway, while strain B204 has the UDP-rhamnose biosynthetic pathway.

**Optimization of Kaempferol 3-*O*-Rhamnoside and Quercetin 3-*O*-Rhamnoidse in *E. coli*.** Using strain B202, production of quercetin 3-*O*-rhamnoside and kaemperol 3-*O*-rhamnoside was optimized. The substrate was added at several time points to monitor its conversion to the reaction product (Figure 4). The final concentrations of kaempferol and quercetin added were 500 $\mu$M (143 mg/L) and 400 $\mu$M

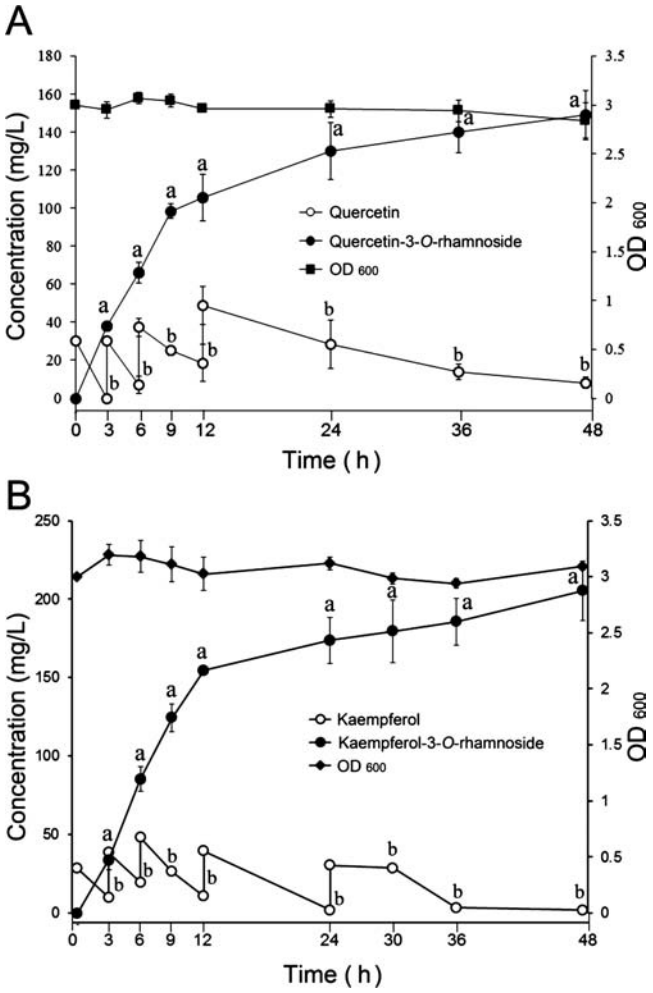


**Figure 4.** Production of (A) quercetin 3-*O*-rhamnoside and (B) kaempferol-3-*O*-rhamnoside using strain B202. After induction of enzymes, the B202 cells were resuspended in 5 mL of M9 medium containing 2% glucose and the cell concentration was adjusted to an $OD_{600}$ of 3. Because of the low solubility of quercetin and kaempferol, these compounds (each at 100 $\mu$M) were added at 0, 3, 6, 12, and 24 h. The final concentration of kaempferol was 500 $\mu$M (143 mg/L), and that of quercetin (100 $\mu$M) was 400 $\mu$M (120.8 mg/L). The reaction mixture (200 $\mu$L) was collected at 3, 6, 9, 12, 24, 36, and 48 h and centrifuged. The supernatant was analyzed using HPLC. Three independent experiments were performed. Different letters indicate a significant difference at $p < 0.01$.

(120.8 mg/L), respectively. The production of each product was monitored for 48 h. The concentration of quercetin 3-*O*-rhamnoside rapidly increased for 9 h, at which time approximately 70% of the added quercetin was converted into quercetin 3-*O*-rhamnoside and approximately 100 mg/L quercetin 3-*O*-rhamnose was produced. After 9 h, the production of quercetin 3-*O*-rhamnose continued to increase, and 150 mg/L quercetin 3-*O*-rhamnoside was produced at 48 h (Figure 4A). Kaempferol 3-*O*-rhamnoside was also rapidly produced until 12 h. At 48 h, approximately 200 mg/L kaempferol 3-*O*-rhamnoside was produced (Figure 4B).

In summary, flavonol 3-*O*-rhamnosides (kaempferol 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside) were synthesized in *E. coli* after introduction of the *AtUGT78D1* gene and quercetin supplementation in the culture. Endogenous dTDP-rhamnose was used as the sugar donor. To increase the production of quercetin 3-*O*-rhamnoside, *RHM2* was co-expressed along with *AtUGT78D1*. The resulting *E. coli* strain produced 60% more quercetin 3-*O*-rhamnoside than the other strains. The yields of kaempferol 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside were approximately 200 and 150 mg/L, respectively.

Natural products from plants have been excellent resources for the development of new medicine and medicinal food. However, complex extraction methods and limited supplies of plant materials have been a major limitation on their wider use. Using microbial systems for the production of natural products could be a good alternative to natural resources. Using *E. coli* expressing flavonol glycosyltransferase and UDP-rhamnose synthase, kaempferol 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside were successfully synthesized. Both of these compounds have shown promising activity against viruses, bacteria, and fungi.[7−9] For example, they might be useful as natural alternatives to antibiotics in livestock.

## AUTHOR INFORMATION

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### Notes

The authors declare no competing financial interest.

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