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SHORT COMMUNICATION

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Production of kaempferol 3-*O*-rhamnoside from glucose using engineered *Escherichia coli*

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Abstract Flavonoids are ubiquitous phenolic compounds and at least 9,000 have been isolated from plants. Most flavonoids have been isolated and assessed in terms of their biological activities. Microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* are efficient systems for the synthesis of flavonoids. Kaempferol 3-*O*-rhamnoside has notable biological activities such as the inhibition of the proliferation of breast cancer cells, the absorption of glucose in the intestines, and the inhibition of the self-assembly of beta amyloids. We attempted to synthesize kaempferol 3-*O*-rhamnoside from glucose in *E. coli.* Five flavonoid biosynthetic genes [tyrosine ammonia lyase (*TAL*), 4-coumaroyl CoA ligase (*4CL*), chalcone synthase (*CHS*), flavonol synthase (*FLS*), and flavonol 3-*O*-rhamnosyltransferase (*UGT78D1*)] from tyrosine were introduced into *E. coli* that was engineered to increase tyrosine production. By using this approach, the production of kaempferol 3-*O*-rhamnoside increased to 57 mg/L.

Keywords Flavonoid glycoside · Kaempferol 3-*O*-rhamnoside · Metabolic engineering

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Introduction

Flavonoids are ubiquitous phenolic compounds, and at least 9,000 have been isolated from plants [21]. Attempts have been made to improve the biological activity of flavonoids by generating structural variations through chemical synthesis [1, 3, 12]. However, the biological activity of flavonoids has been mainly evaluated in flavonoids isolated from plants. Elucidation of flavonoid biosynthetic pathways and the characterization of genes involved in these pathways may facilitate in the synthesis of flavonoids in microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* [10, 13, 15, 23, 25]. Furthermore, several studies have investigated *E. coli* mutants that could supply the building blocks for flavonoid biosynthesis [7, 19].

Naringenin, which is synthesized from one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA by chalcone synthase, is a precursor of numerous flavonoids. Even in plants, naringenin is converted into flavones (apigenin, luteolin), flavonols (kaempferol and quercetin), or isoflavones (genistein and daidzein). The genes for the synthesis of these flavones, flavonols, and isoflavones have been isolated and characterized [22, 24]. Therefore, with the accumulated information about downstream genes in flavonoid biosynthesis pathways and the development of *E. coli* metabolic engineering, it is possible to synthesize biologically active flavonoids from glucose. In this report, we attempted to synthesize kaempferol 3-*O*-rhamnoside from glucose in *E. coli* because this compound has notable biological activities. Kaempferol 3-*O*-rhamnoside has been shown to inhibit the proliferation of breast cancer cells and the absorption of dietary glucose in the intestines [2, 18]. In addition, kaempferol 3-*O*-rhamnoside was shown to protect against beta-amyloid induced cell death by inhibiting the self-assembly of beta amyloids [20]. Therefore,

Table 1 Plasmids, *Escherichia coli* strains, and primers used in this study

Plasmids or *E. coli* strain	Relevant properties or genetic marker	Source or reference
Plasmids		
pACYCDuet	P15A ori, Cm^r	Novagen
pCDFDuet	CDF ori, Str^r	Novagen
pGEX	pBR322 ori, Amp^r	GE Healthcare
pA-SeTAL	pACYCDuet carrying *TAL* from *Saccharothrix espanaensis*	Kim et al. [7]
pA-tyrA-aroG	pACYCDuet carrying *aroG* and *tyrA* from *E. coli*	Kim et al. [7]
pA-aroG-SeTAL-tyrA	pACYCDuet carrying *TAL* from *S. espanaensis, aroG* and *tyrA* from *E. coli*	Kim et al. [7]
pA-aroG^{fbr}-ppsA-tktA-SeTAL-tyrA^{fbr}	pACYCDuet carrying *TAL* from *S. espanaensis, aroG*^{fbr}, *PPSA, tktA,* and *tyrA*^{fbr} from *E. coli*	Kim et al. [7]
pC-Os4CL-PeCHS	pCDFDuet carrying *4CL* from *O. sativa* and PeCHS from *Populus euramericana*	Kim et al. [7]
pE-PeFLS-AtD1	pETDuet carrying FLS from *Populus euramericana* and AtUGT78D1 from *Arabidopsis thaliana*	This study
Strains		
BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm lon* (DE3)	Novagen
B-TPI	BL21(DE3) *ΔtyrR::FRT- ΔPheA:: ΔicdA-FRT FRT-kan*^{R}*-FRT*	Kim et al. [7]
B-KR1	BL21 (DE3) carrying pA-SeTAL, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1	This study
B-KR2	BL21 (DE3) carrying pA-aroG-SeTAL-tyrA, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1	This study
B-KR3	BL21 (DE3) carrying pA-aroG^{fbr}-SeTAL-tyrA^{fbr}, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1	This study
B-KR4	BL21 (DE3) carrying pA-aroG^{fbr}-ppsA-tktA-SeTAL-tyrA^{fbr}, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1	This study
B-KR5	B-TPI carrying pA-aroG^{fbr}-ppsA-tktA-SeTAL-tyrA^{fbr}, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1	This study

kaempferol 3-*O*-rhamnoside could serve as a potential treatment of cancer and Alzheimer's disease. By introducing a kaempferol 3-*O*-rhamnoside biosynthesis pathway and engineering a tyrosine biosynthesis pathway in *E. coli*, we have successfully synthesized approximately 57 mg/L of kaempferol 3-*O*-rhamnoside from glucose. To the best of our knowledge, this is the first report about de novo synthesis of flavonoid glycoside from glucose in *E. coli*.

Materials and methods

Plasmid constructs and *E. coli* strains

Tyrosine ammonia lyase *(TAL)* was cloned from *Saccharothrix espanaensis* (ATCC 51144) [7]. 4-Coumaroyl-CoA ligase (*Os4CL*) and chalcone synthase (*PeCHS*) were previously cloned from *Oryza sativa* [9] and *Populus euramericana* Guinier [6], respectively. The *FLS* gene (*PeFLS*) was previously cloned from poplar [4] and subcloned into the *Nde*I/*Kpn*I site of a pETDuet vector. *AtUGTD1*, which was also previously cloned [5], was subcloned into the *Eco*RI/*Not*I sites of pETDuet vector containing *FLS*. Other constructs used in this study were cloned previously and were listed in Table 1.

An *E. coli* mutant was made previously using the Quick and Easy Conditional Knockout Kit (Gene Bridges, Heidelberg, Germany) [7].

Production of kaempferol 3-*O*-rhamnoside from glucose in *E. coli*

To compare the effects of different constructs on the production of kaempferol 3-*O*-rhamnoside, three constructs were transformed into *E. coli* BL21 (DE3). We produced four experimental strains (strain B-KR1 to B-KR4) by transforming *E. coli* BL21 (DE3) with both the constructs, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1, which differed by having either pA-SeTAL (strain B-KR1), pA-aroG-SeTAL-tyrA (strain B-KR2), pA-aroG^{fbr}-SeTAL-tyrA^{fbr} (strain B-KR3), or pA-aroG^{fbr}-ppsA-tktA-SeTAL-tyrA^{fbr} (strain B-KR4) (Table 1). Each *E. coli* strains (B-KR1 to B-KR4) were grown in Luria-Bertan (LB) broth containing proper antibiotics at 50 μg/mL for 18 h. The culture was inoculated into fresh LB medium containing antibiotics and the cells were grown until the OD_{600} exceeded 1.0. The cells were collected by centrifugation and then washed briefly with M9 medium (Amresco, Solon, Ohio, USA). The cell concentration was adjusted to an OD_{600} of 1.0 with 2 mL of M9 medium supplemented with 1 % yeast extract,

2 % glucose, 50 μg/mL antibiotics, and 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). The mixture was incubated at 30 °C for 48 h and then 200 μL of the culture medium was extracted with 600 μL of ethyl acetate, dried, and dissolved in 50 μL of dimethylsulfoxide (DMSO). The reaction product was analyzed using a Varian high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) equipped with a photodiode array (PDA) detector and a Polaris 5 C18-A column (250 mm × 4.6 mm, Agilent Technologies). The mobile phase consisted of 0.1 % formic acid in water and acetonitrile. The program was as follows: 20 % acetonitrile at 0 min, 45 % acetonitrile at 10 min, 80 % acetonitrile at 20 min, 90 % acetonitrile at 20.1 min, 20 % acetonitrile at 25.1, and 20 % acetonitrile at 30 min. The flow rate was 1 mL/min and UV absorbance was simultaneously detected at wavelengths of 290 and 340 nm.

To compare the production of kaempferol 3-*O*-rhamnoside in *E. coli* BL21(DE3) and B-TPI, three constructs, pA-aroG^{fbr}-ppsA-tktA-seTAL-tyrA^{fbr}, pC-Os4CL-PeCHS4, and pE-PeFLS-AtD1 were transformed into *E. coli* BL21(DE3) and B-TPI, respectively. The resulting strains, B-KR4 and B-KR5 (Table 1), were grown as described above and the rate of production of kaempferol 3-*O*-rhamnoside in these two strains was determined after 48 h.

To monitor the production of kaempferol 3-*O*-rhamnoside using B-KR5 (Table 1), B-KR5 was grown as described above. Cells were then harvested and resuspended in 10 mL of M9 medium containing 1 % yeast extract, 2 % glucose, and 1 mM IPTG and 50 μg/mL of antibiotics. The cell concentration was adjusted to $OD_{600} = 1$. Samples were collected at 4, 8, 12, 24, 30, and 48 h and were analyzed as described above.

The mean and standard error of the mean were calculated from triplicate experiments. An analysis of variance (ANOVA) was carried out with a Tukey's test of the pairwise comparisons of the experimental strains with a significance level of $P = 0.01$ using Excel 2010 (Microsoft Corp., Redmond, WA, USA).

Results and discussion

Production of kaempferol 3-*O*-rhamnoside from glucose

Kaempferol 3-*O*-rhamnoside is synthesized from the primary metabolite tyrosine. *E. coli* does not possess the pathway for kaempferol 3-*O*-rhamnoside biosynthesis from tyrosine. Therefore, to synthesize kaempferol 3-*O*-rhamnoside from tyrosine, flavonoid biosynthetic genes should be introduced into *E. coli*. Kaempferol 3-*O*-rhamnoside was synthesized from tyrosine by the sequential reaction of five genes, *TAL*, *4CL*, *CHS*, *FLS*, and *AtUGT78D1* as shown in Fig. 1. TAL converts tyrosine into *p*-coumaric acid, which is converted into *p*-coumaroyl-CoA by the action of 4CL. *p*-Coumaroyl-CoA along with three molecules of malonyl-CoA, which are natively synthesized by *E. coli*, are converted into naringenin by CHS. This naringenin is then converted into kaempferol by the action of FLS. The final reaction in which kaempferol attaches a rhamnose at the 3-hydroxy group is mediated by AtUGT78D1.

We cloned these five genes in three different vectors, each of which had a different selection marker and replication origin. *PeFLS* and *AtUGT78D1*, which were located downstream in the synthetic pathway, were cloned into the highest copy number plasmid, pETDuet-1. The PeFLS used in this study has a higher catalytic efficiency than that from *A. thaliana* and *Camellia sinensis* [4]. And, AtUGT78D1 has a specificity for U(T)DP-rhamnose and does not use other nucleotide sugars such as U(T)DP-glucose [27]. The *4CL* and *CHS* genes were cloned into the second highest copy number plasmid, pCDFDuet-1, which occurs at 20–40 copies in each *E. coli* cell. Finally, *TAL* was cloned into the lowest copy number plasmid, pACYCDuet-1, of which only 10–12 copies are present in each *E. coli* cell. We used this strategy because more of the downstream protein was needed to prevent the accumulation of the reaction intermediates. The three constructs containing the five genes were transformed into *E. coli* BL21(DE3). The HPLC analysis of the culture filtrate showed five peaks (P1–P4 and M in Fig. 2b). Based on the UV-absorbance, P1 at 8.49 min was likely to be bis-noryangonin (BNY), which is produced as by-products of the reaction catalyzed by CHS. The molecular mass of P2 at 8.96 min was 431-Da in the negative mode (Fig. 2b, d), which corresponded to the molecular mass of kaempferol 3-*O*-rhamnoside. Additionally, P2 was indistinguishable with the standard compound, kaempferol 3-*O*-rhamnoside in the UV absorbance, HPLC retention time, and MS/MS pattern (Fig. 2a, c, d). From measurements of UV-absorbance in previous study [16], the P3 at 9.62 min was likely to be naringenin chalcone.

Notably, kaempferol was not detected. Therefore, it is likely that any kaempferol was immediately converted into kaempferol 3-*O*-rhamnoside, indicating that the amount of nucleotide-rhamnose was not a limiting factor. Conversely, naringenin (P4 at 12.82 min) was observed, indicating that the reaction catalyzed by FLS might be a rate-limiting step. These results indicated that *E. coli* harboring five kaempferol 3-*O*-rhamnoside biosynthetic genes successfully synthesized kaempferol 3-*O*-rhamnoside.

Overexpression of four genes to increase the production of kaempferol 3-*O*-rhamnoside

As shown in Fig. 1, tyrosine is a precursor of *p*-coumaric acid and is synthesized via the shikimate pathway in

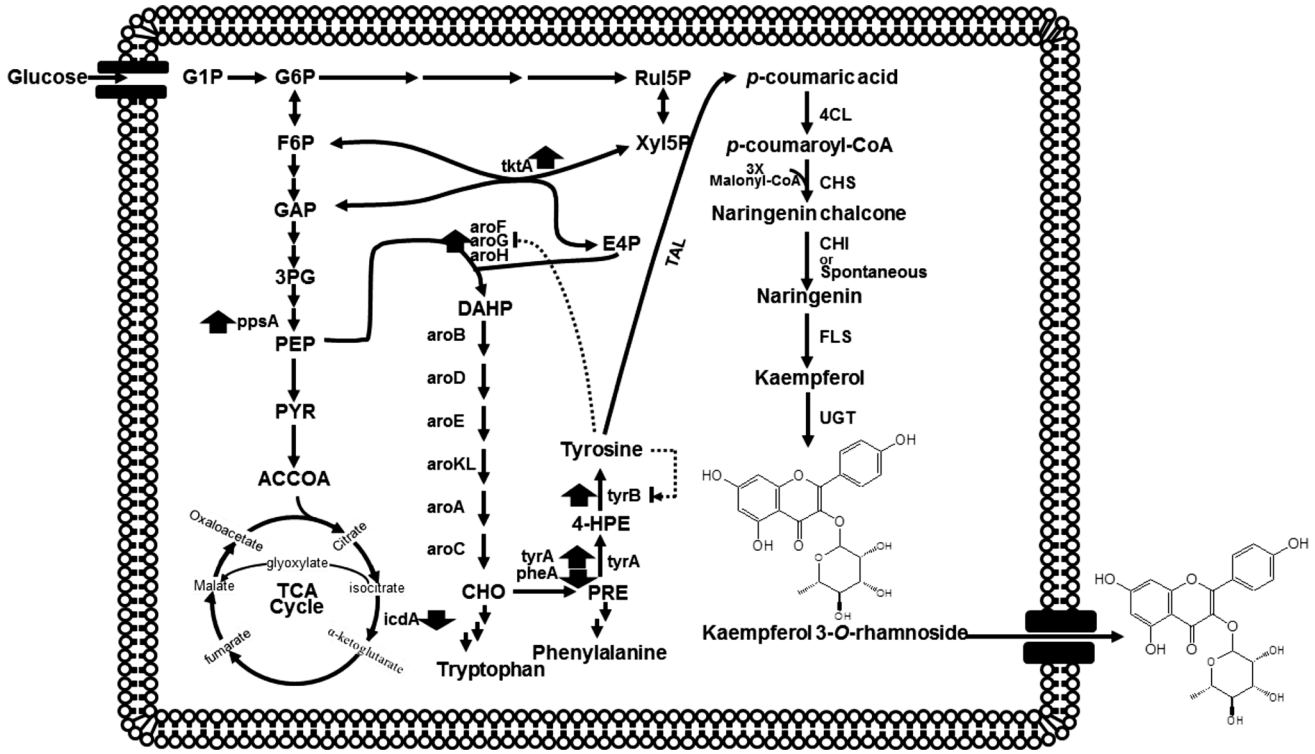

Fig. 1 A schematic pathway for the production of kaempferol 3-*O*-rhamnoside from glucose in *Escherichia coli*. *G1P* glucose 1-phosphate, *G6P* glucose 6-phosphate, *F6P* fructose 6-phosphate, *GAP* glyceraldehyde 3-phosphate, *3PG* 3-phosphoglycerate, *PEP* phosphoenolpyruvate, *PYR* pyruvate, *ACCOA* acetyl-CoA, *Rul5P* ribulose 5-phosphate, *Xyl5P* xylose 5-phosphate, *E4P* erythrose 4-phosphate, *DAHP* 3-deoxy-D-arabino-heptulosonate-7-phosphate, *CHO* chorismate, *PRE* prephenate, *4-HPE* 4-hydroxyphenylpyruvate, *aroB* 3-dehydroquinate synthase, *aroD* 3-dehydroquinate dehydratase, *aroE* shikimate dehydrogenase, *aroKL* shikimate kinase I, II, *aroA* 3-phosphoshikimate-1-carboxyvinyltransferase, *aroC* chorismate synthase, *tyrA* chorismate mutase/prephenate dehydrogenase, *pheA*, prephenate dehydratase, *tyrB* phenylalanine aminotransferase, *TAL* tyrosine amino lyase, *4CL* 4-coumaroyl-CoA ligase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *FLS* flavonol synthase, *UGT* kaempferol 3-*O*-rhamnosyltransferase

E. coli. Four genes, *ppSA*, *tktA*, *aroG*, and *tyrA*, are critical for increasing the pool of tyrosine. PpSA converts pyruvate into phosphoenolpyruvate and TktA produces erythrose 4-phosphate from fructose 6-phosphate and glyceraldehyde 3-phosphate [13, 17, 26]. Using phosphoenolpyruvate and erythrose 4-phosphate, AroG synthesizes 3-deoxy-D-arabino-heptulosonate 7-phosphate, which is the first product of the shikimate pathway. TyrA catalyzes the conversion of prephenate into 4-hydroxyl-phenylpyruvate. While AroG and TyrA are inhibited by tyrosine, AroG^{fbr} and TyrA^{fbr} are respective mutant forms that are not inhibited by tyrosine [13]. Therefore, we tested the constructs that contained four different combinations of genes to evaluate the effect of the production of tyrosine on the production of kaempferol 3-*O*-rhamnoside. The first construct (pA-SeTAL) encodes only TAL, which uses only endogenous tyrosine. The second construct (pA-aroG-SeTAL-tyrA) overexpressed *aroG* and *tyrA* in addition to *SeTAL*. In the third construct (pA-aroG^{fbr}-seTAL-tyrA^{fbr}), feedback inhibition free *aroG* and *tyrA* (*aroG*^{fbr} and *tyrA*^{fbr}) were overexpressed. In the fourth construct (pA-aroG^{fbr}-ppsA-tktA-seTAL-tyrA^{fbr}), *ppsA* and *tktA* along with *aroG*^{fbr} and *tyrA*^{fbr} were overexpressed. Each construct was transformed into *E. coli* BL21(DE3) along with pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1, and the transformants were designated B-KR1, B-KR2, B-KR3, and B-KR4 (Table 1). The production of kaempferol 3-*O*-rhamnoside from glucose in each strain was measured (Fig. 3). Strain B-KR1 produced approximately 5.5 mg/L of kaempferol 3-*O*-rhamnoside. Strain B-KR2, which harbored *aroG* and *tyrA*, produced 9 mg/L of kaempferol 3-*O*-rhamnoside. Strain B-KR3 produced a much greater amount of kaempferol 3-*O*-rhamnoside (23 mg/L) than either B-KR1 or B-KR2. The highest amount of kaempferol 3-*O*-rhamnoside was produced by B-KR4 (27 mg/L), which is approximately five times more than that produced by B-KR1. Therefore, increasing the amount of tyrosine by overexpressing several genes in the shikimate pathway contributed to an increase in the production of kaempferol 3-*O*-rhamnoside, which agreed with our previous results on the production of *O*-methylated flavonoids [7].

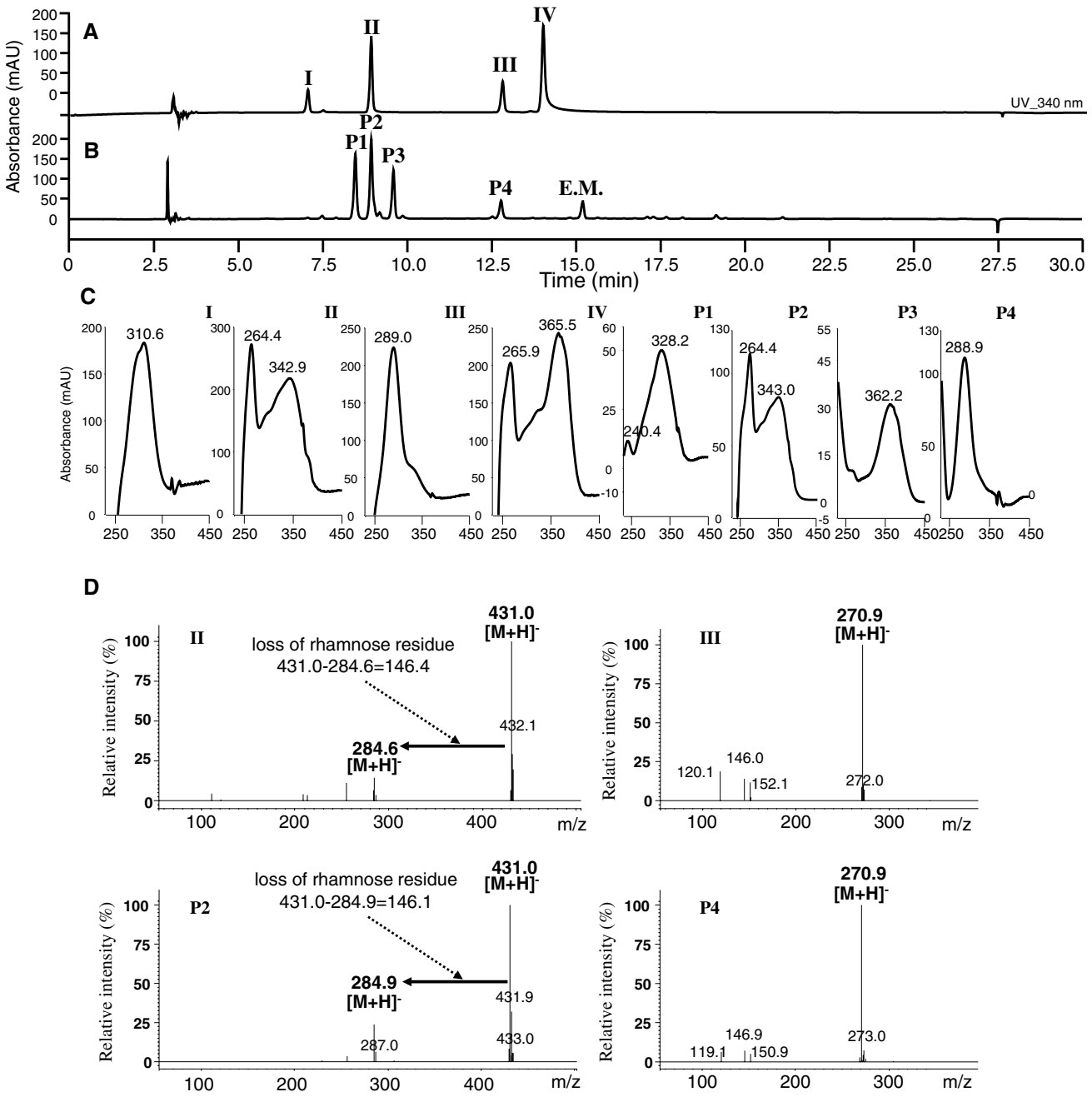

Fig. 2 HPLC analysis of reaction product. **a** HPLC chromatogram of authentic compounds. *I p*-coumaric acid, *II* kaempferol 3-*O*-rhamnoside, *III* naringenin, *IV* kaempferol. **b** HPLC analysis of reaction products from strain B-KR1. *P1* is likely to be a bis-noryangonin (BNY). *P2* is kaempferol 3-*O*-rhamnoside, *P3* is likely to be naringenin chalcone and *P4* was determined to be naringenin. *M* is likely to be *E. coli* metabolite, which was also observed in the *E. coli* strain containing empty vectors. **c** UV spectrums of authentic compounds and reaction products produced by K-KR1. **d** MS/MS analysis of the reaction product 2 and 3. **a** HPLC analysis of reaction products from strain B-KR1. *M* indicates *E. coli* metabolite and *P1* indicates kaempferol 3-*O*-rhamnoside. **b** MS/MS analysis of standard kaempferol 3-*O*-rhamnoside (*II*), naringenin (*III*), and reaction products (*P2* and *P4*)

Engineering of *E. coli* to improve the production of kaempferol 3-*O*-rhamnoside

E. coli was engineered to improve the production of kaempferol 3-*O*-rhamnoside. The B-TPI strain, which was previously used for the production of *O*-methylated flavonoids [7] was also used. Three genes, *tyrR*, *pheA,* and *icdA* were deleted from *E. coli* BL21(DE3) to generate the strain B-TPI. *tyrR* encoded a transcription factor that regulates the first step of the shikimate pathway, whereas the end product,

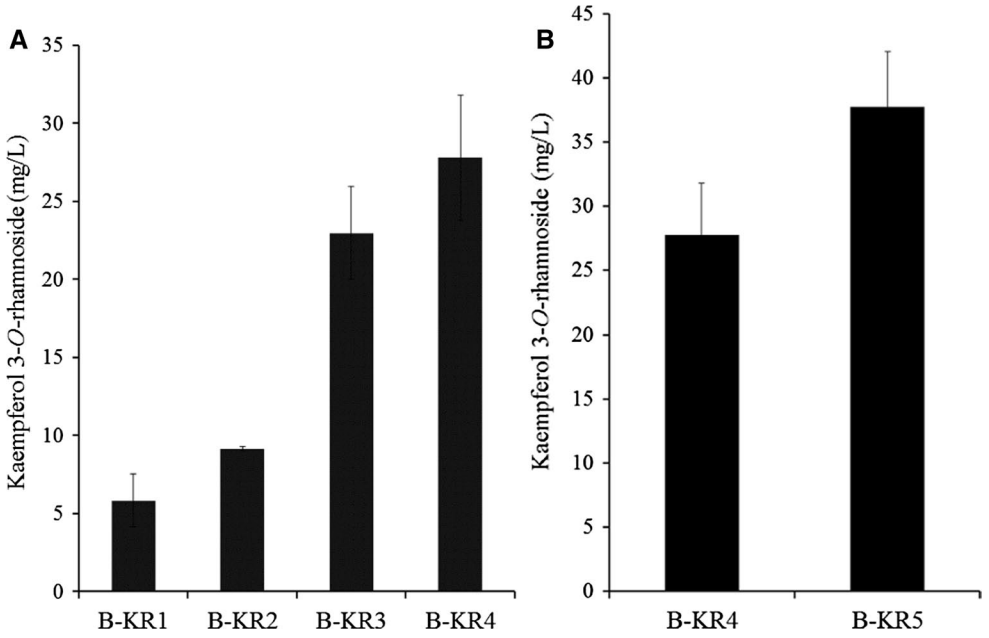

Fig. 3 Effect of different gene combinations (**a**) and *E. coli* mutants (**b**) on the production of kaempferol 3-*O*-rhamnoside. B-KR1, BL21 (DE3) carrying pA-SeTAL, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1; B-KR2, BL21 (DE3) carrying pA-aroG-seTAL-tyrA, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1; B-KR3, BL21 (DE3) carrying pA-aroG^{fbr}-seTAL-tyrA^{fbr}, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1; B-KR4, BL21 (DE3) carrying pA-aroG^{fbr}-ppsA-tktA-seTAL-tyrA^{fbr}, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1; B-KR5, B-TPI carrying pA-aroG^{fbr}-ppsA-tktA-seTAL-tyrA^{fbr}, pC-Os4CL-PeCHS4 and pE-PeFLS-AtD1. *Error bars* indicate mean values ± SD from three independent experiments

tyrosine inhibits TyrR, which results in inhibition of shikimate pathway. *pheA* encodes chorismate mutase and prephenate dehydratase, which competes with TyrA for prephenate. Coenzyme A (CoA) is required for the synthesis of *p*-coumaroyl-CoA and malonyl-CoA, which are substrates of CHS. Therefore, a sufficient supply of CoA is critical for the production of kaempferol 3-*O*-rhamnoside. IcdA, which converts isocitrate into α-ketoglutarate, was also deleted. The deletion of *icdA* in *E. coli* has been shown to provide more CoA for the synthesis of *p*-coumaroyl-CoA [8]. This explains why strain B-TPI had a high rate of naringenin production, which is a substrate of FLS. This strain was transformed using three constructs, pA-aroG^{fbr}-ppsA-tktA-seTAL-tyrA^{fbr}, pC-Os4CL-PeCHS4, and pE-PeFLS-AtD1, and the resulting strain was designated as B-KR5. B-KR5 produced approximately 37 mg/L of kaempferol 3-*O*-rhamnoside after 24 h of incubation, whereas strain B-KR4 produced only 28 mg/L (Fig. 3b). Therefore, deletion of the three genes was critical for the increase in the yield of kaempferol 3-*O*-rhamnoside.

The incubation temperature, glucose concentration, and cell concentration were optimized using B-KR5. Three temperatures, 25, 30, and 37 °C were tested; 30 °C produced the highest production rate (~38 mg/L), followed by 25 °C (~34 mg/L) and 37 °C (~6 mg/L). Cell concentration was adjusted to 0.5, 1, and 2 at OD_{600}. At $OD_{600} = 1$, the production of kaempferol 3-*O*-rhamnoside was highest

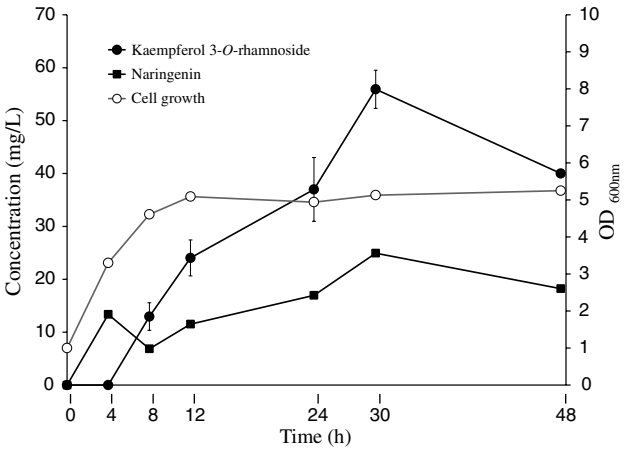

Fig. 4 Production of kaempferol 3-*O*-rhamnoside by strain B-KR5. *Error bars* indicate mean values ± SD from three independent experiments

(38 mg/L), followed by 0.5 (23 mg/L), and 2 (6 mg/L). Using strain B-KR5, we monitored the production of kaempferol 3-*O*-rhamnoside. As determined above, the initial cell concentration was 1 at OD_{600}. During the first 4 h, no production of kaempferol 3-*O*-rhamnoside was detected. After 4 h, its production continued to increase, showing a concentration of 58 mg/L after 30 h (Fig. 4).

We have synthesized kaempferol 3-*O*-rhamnoside by reconstructing the flavonoid biosynthetic pathway in *E. coli*. Previously, kaempferol 3-*O*-rhamnoside was synthesized by biotransformation of kaempferol using *E. coli* transformed *AtUGTD1* and *RHM2* [5]. RHM catalyzes the conversion of uridine diphosphate glucose (UDP-glucose) into UDP-rhamnose. The yield of kaempferol 3-*O*-rhamnoside was approximately 200 mg/L, which was about threefold higher than by synthesizing it from glucose. Because the price of kaempferol is much higher than glucose, the current study has an advantage over biotransformation.

The tyrosine content was critical for the production of kaempferol 3-*O*-rhamnoside. The four different constructs that we tested differed in the capacity to produce tyrosine that they conferred on the host cell. Strain B-KR1 did not overexpress any gene involved in tyrosine biosynthesis. Strain B-KR2 overexpressed *aroG* and *tyrA*, whereas strain B-KR3 overexpressed the mutant versions of *aroG* and *tyrA* (*aroG*^{fbr} and *tyrA*^{fbr}), either of which is not inhibited by the end product, tyrosine. Furthermore, strain B-KR4 overexpressed *ppsA* and *tktA*, in addition to *aroG*^{fbr} and *tyrA*^{fbr} in order to supply more substrate for AroG^{fbr}. Therefore, strain B-KR4 was expected to produce more tyrosine than the other strains [13, 14]. The final yield of kaempferol 3-*O*-rhamnoside correlated with the production of tyrosine and varied from 6 mg/L in strain B-KR1 to 28 mg/L in B-KR4.

E. coli synthesizes dTDP-rhamnose, but not UDP-rhamnose. In our current study, endogenous dTDP-rhamnose was used. AtUGT78D1 uses both dTDP-rhamnose and UDP-rhamnose, although it has higher affinity for UDP-rhamnose [11]. In the previous study [5], we overexpressed *RHM* to generate UDP-rhamnose. However, the final yield of kaempferol 3-*O*-rhamnoside did not change compared to the yield of *E. coli*, which did not contain *RHM2*. The amount of the sugar-acceptor kaempferol that was synthesized by introducing this pathway into *E. coli* was likely to be much less than the amount of UDP-rhamnose and endogenous dTDP-rhamnose. Therefore, due to the lack of this sugar acceptor, the yield of kaempferol 3-*O*-rhamnoside did not increase despite the introduction of excessive UDP-rhamnose. Therefore, a balance between sugar donor (UDP- or dTDP-rhamnose) and sugar acceptor (kaempferol) is essential in increasing the final yield of kaempferol 3-*O*-rhamnoside. This finding was also supported by our previous study, in which kaempferol 3-*O*-rhamnoside production increased when the gene *RHM2* was overexpressed and kaempferol was supplied [5].

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