Production of 7-O-Methyl Aromadendrin, a Medicinally Valuable Flavonoid, in *Escherichia coli*

Sailesh Malla,^a Mattheos A. G. Koffas,^b Romas J. Kazlauskas,^c and Byung-Gee Kim^a

Laboratory of Molecular Biotechnology and Biomaterials, School of Chemical and Biological Engineering, Seoul National University, Seoul, South Korea^a; Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York, USA^b; and Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota, USA^c

7-O-Methyl aromadendrin (7-OMA) is an aglycone moiety of one of the important flavonoid-glycosides found in several plants, such as *Populus alba* and *Eucalyptus maculata*, with various medicinal applications. To produce such valuable natural flavonoids in large quantity, an *Escherichia coli* cell factory has been developed to employ various plant biosynthetic pathways. Here, we report the generation of 7-OMA from its precursor, *p*-coumaric acid, in *E. coli* for the first time. Primarily, naringenin (NRN) (flavanone) synthesis was achieved by feeding *p*-coumaric acid and reconstructing the plant biosynthetic pathway by introducing the following structural genes: 4-coumarate–coenzyme A (CoA) ligase from *Petroselinum crispum*, chalcone synthase from *Petunia hybrida*, and chalcone isomerase from *Medicago sativa*. In order to increase the availability of malonyl-CoA, a critical precursor of 7-OMA, genes for the acyl-CoA carboxylase α and β subunits (*nfa9890* and *nfa9940*), biotin ligase (*nfa9950*), and acetyl-CoA synthetase (*nfa3550*) from *Nocardia farcinica* were also introduced. Thus, produced NRN was hydroxylated at position 3 by flavanone-3-hydroxylase from *Arabidopsis thaliana*, which was further methylated at position 7 to produce 7-OMA in the presence of 7-O-methyltransferase from *Streptomyces avermitilis*. Dihydrokaempferol (DHK) (aromadendrin) and sakuranetin (SKN) were produced as intermediate products. Overexpression of the genes for flavanone biosynthesis and modification pathways, along with malonyl-CoA overproduction in *E. coli*, produced 2.7 mg/liter (8.9 μ M) 7-OMA upon supplementation with 500 μ M *p*-coumaric acid in 24 h, whereas the strain expressing only the flavanone modification enzymes yielded 30 mg/liter (99.2 μ M) 7-OMA from 500 μ M NRN in 24 h.

Flavonoids are the most ubiquitous polyphenolic secondary metabolites produced by plants. Depending on the environmental conditions, they accumulate in all organs and tissues of plants at different developmental stages. More than 8,000 naturally occurring flavonoids have been identified, and many of them are common in higher plants (10, 39). These compounds are a diverse group of phytochemicals fulfilling various functions, such as antioxidant, anti-inflammatory, antitumor, antiarteriosclerotic, antiproliferative, estrogenic, or antiestrogenic activities (26, 30, 31). These functional variations result mainly from the structural diversities that originate from various chemical modifications, such as hydroxylation, methylation, methoxylation, glycosylation, acylation, and prenylation.

7-O-Methyl aromadendrin (7-OMA) (also known as aromadendrin 7-methyl ether or 7-O-methyl dihydrokaempferol [DHK]) (Fig. 1) is a plant-based pharmaceutically important flavonoid compound with anti-inflammatory activity (24), anticancer activity, an apoptosis effect (6), etc. The compound has also been reported to stimulate insulin-mediated glucose uptake on liver carcinoma cells and adipocytes *in vitro*, implying the compound is a potential candidate to improve the diabetic conditions in type 2 diabetes mellitus (DM) (44). 7-OMA has been isolated from several medicinally important plants, including *Populus alba* (35), *Eupatorium* spp. (11), *Artemisia campestris* (12), *Artemisia dracunculus* (2), *Inula viscosa* L. (42), and *Eucalyptus maculata* (1).

In plants, the biosynthesis of flavonoids is initiated by the enzyme 4-coumarate–coenzyme A (CoA) ligase (4CL) converting *p*-coumaric acid into its coenzyme A ester (22). Then, one molecule of *p*-coumaroyl-CoA condenses with three molecules of malonyl-CoA in the presence of chalcone synthase (CHS), leading

to the production of naringenin (NRN) chalcone (17), which is further isomerized to (2S)-flavanone by chalcone flavanone isomerase (CHI) (28) (Fig. 1). The (2S)-flavanone is the first flavonoid molecule from which the biosynthetic pathway diverges into several side branches, resulting in a class of various flavonoids.

Despite the fact that plants are the natural sources of flavonoids, the large-scale production of flavonoids by plant extraction for nutraceutical use and drug development is a challenge. Similarly, tissue culture or chemical synthesis is also far from a practical way to produce flavonoids and their derivatives (25, 37). Alternatively, the assembly of the entire biosynthetic pathway into a simple microbe, such as *E. coli*, and metabolic engineering of the resulting recombinant microbial strain have been demonstrated to be a promising alternative for the production of large quantities of various flavonoid molecules. Many of the structural genes from plants, fungi, and bacteria have been cloned and expressed in *E. coli* and/or *Saccharomyces cerevisiae* for efficient production of flavonoid compounds (13, 19, 43). Furthermore, incorporation of the regiospecific posttailoring enzymes leads to the synthesis of target natural molecules, as well as unnatural derivatives with desired modifications.

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Address correspondence to Byung-Gee Kim, byungkim@snu.ac.kr.

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FIG 1 Proposed biosynthetic pathways for 7-OMA starting from *p*-coumaric acid.

Although a number of studies have been carried out to improve production of flavanones and their derivatives, the production of 7-OMA in a microbial system has not been reported. Here, we report the production of 7-OMA, starting from its precursor, *p*-coumaric acid, in *Escherichia coli*. Most of the proteins from *Nocardia* strains were well expressed in the *E. coli* system (unpublished results), and these strains also produce various macrolide polyketide compounds. Therefore, in order to increase the intracellular malonyl-CoA pool in *E. coli*, we redesigned the acetate-assimilating and malonyl-CoA synthetic pathways using the enzymes from *Nocardia farcinica*. In addition, in order to explore the possibility of further modifying NRN, i.e., flavanone, into other valuable flavonoid compounds, the biosynthesis of 7-OMA was undertaken by introducing enzymes for performing C-3 hydroxylation and 7-O-methylation. Our results suggest that an intermediate NRN-feeding strategy is still much more advantageous than complete biosynthesis of flavonoid derivatives from *p*-coumaric acid.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains, vectors, and plasmids used in this study are listed in Table 1. *E. coli* DH5 α (Invitrogen, Seoul, South Korea) was used for plasmid cloning and propagation, while *E. coli* BL21(DE3) was used for flavonoid production. Vectors pACYCDuet-1, pETDuet-1, pCDFDuet-1, pRSFDuet-1 (Novagen), and pET-24ma(+), donated by Hiroshi Sakamoto (Pasteur Institute, France), were used for cloning and subcloning. The pGEM-T system (Promega) was used as a vector for cloning PCR fragments and sequencing. The construction of plasmids pC-*Pc4cl2* (21) and pE-*Phchs*-*Msch* (19) was previously reported.

Culture media and chemicals. *E. coli* was grown in Luria-Bertani (LB) broth or on an agar plate supplemented with the appropriate amount of antibiotics (100 μ g/ml ampicillin, 25 μ g/ml chloramphenicol, 50 μ g/ml streptomycin/spectinomycin, and 35 μ g/ml kanamycin), when necessary, for the selection or maintenance of the plasmids. For flavonoid production, LB medium and slightly modified M9 minimal medium (per liter, 6 g Na₂HPO₄, 3 g K₂HPO₄, 0.5 g NaCl, 0.1% NH₄Cl, 1% glucose, 1 mM MgSO₄ · 7H₂O, 100 μ M CaCl₂, 1.46 mg biotin, 1 mg thiamine-HCl) were

TABLE 1 Bacterial strains and plasmids used in the study

Strain/plasmid	Description	Source/reference
<i>E. coli</i> strains		
DH5 α	General cloning host	Invitrogen
BL21(DE3)	<i>ompT hsdT hsdS</i> ($r_B^- m_B^-$) <i>gal</i> (DE3)	Novagen
E2	BL21(DE3) carrying pC- <i>Pc4cl2</i> and pE- <i>Phchs-Mschi</i>	19
E3	BL21(DE3) carrying pC- <i>Pc4cl2</i> , pE- <i>Phchs-Mschi</i> , and pACYC104	This study
E3FH	BL21(DE3) carrying pC- <i>Atf3h-Pc4cl2</i> , pE- <i>Phchs-Mschi</i> , and pACYC104	This study
E3MT	BL21(DE3) carrying pC- <i>Pc4cl2</i> , pE- <i>Phchs-Mschi</i> , pRSF- <i>Saomt</i> , and pACYC104	This study
E3FHT	BL21(DE3) carrying pC- <i>Atf3h-Pc4cl2</i> , pE- <i>Phchs-Mschi</i> , pRSF- <i>Saomt</i> , and pACYC104	This study
ACYC104	BL21(DE3) carrying pACYC104	This study
F3H	BL21(DE3) carrying pC- <i>Atf3h</i>	This study
OMT	BL21(DE3) carrying pRSF- <i>Saomt</i>	This study
F3H-OMT	BL21(DE3) carrying pC- <i>Atf3h</i> and pRSF- <i>Saomt</i>	This study
Plasmid vectors		
pETDuet-1	Double T7 promoters; ColE1 ori Amp ^r	Novagen
pCDFDuet-1	Double T7 promoters; CloDF13 ori Sm ^r	Novagen
pACYCDuet-1	Double T7 promoters; P15A ori Cm ^r	Novagen
pRSFDuet-1	Double T7 promoters; RSF ori Km ^r	Novagen
pET24ma(+)	Expression vector with T7 promoter; p15A ori Km ^r	Pasteur Institute, France
pE- <i>Phchs-Mschi</i>	pET-Duet-1 carrying <i>chs</i> from <i>P. hybrida</i> and <i>chi</i> from <i>M. sativa</i>	19
pC- <i>Pc4cl2</i>	pCDF-Duet-1 carrying <i>4cl-2</i> from <i>P. crispum</i>	21
pC- <i>Atf3h</i>	pCDF-Duet-1 carrying AT3G51240 from <i>A. thaliana</i>	This study
pC- <i>Atf3h-Pc4cl2</i>	pCDF-Duet-1 carrying AT3G51240 from <i>A. thaliana</i> and <i>4cl-2</i> from <i>P. crispum</i>	This study
pACYC101	pACYCDuet-1 carrying <i>nfa9890</i> from <i>N. farcinica</i>	This study
pACYC102	pACYCDuet-1 carrying <i>nfa9890</i> and <i>nfa9950</i> from <i>N. farcinica</i>	This study
pACYC103	pACYCDuet-1 carrying <i>nfa9890</i> , <i>nfa9950</i> , and T7-rbs- <i>nfa3550</i> from <i>N. farcinica</i>	This study
pACYC104	pACYCDuet-1 carrying <i>nfa9890</i> , T7-rbs- <i>nfa9940</i> , <i>nfa9950</i> , and T7-rbs- <i>nfa3550</i> from <i>N. farcinica</i>	This study
pET24- <i>nfa9940</i>	pET-24ma(+) carrying <i>nfa9940</i> from <i>N. farcinica</i>	This study
pET24- <i>nfa3550</i>	pET-24ma(+) carrying <i>nfa3550</i> from <i>N. farcinica</i>	This study
pRSF- <i>Saomt</i>	pRSFDuet-1 carrying <i>Saomt</i> from <i>S. avermitilis</i>	This study

used throughout. Authentic malonyl-CoA, NRN, and *p*-coumaric acid were purchased from Sigma-Aldrich Co. (South Korea).

DNA manipulations. Recombinant DNA techniques were performed according to standard procedures (33). Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs and Promega. Total RNA from *Arabidopsis thaliana* was isolated using an RNeasy Minikit (Qiagen) according to the manufacturer's instructions. The cDNA of *A. thaliana* was obtained by reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega), and PCR was carried out with *LaTaq* polymerase (Takara). Oligonucleotide primers were from Cosmo Co. Ltd. (South Korea). All the PCR primers used in this study are described in Table S1 in the supplemental material.

Plasmid construction. Based on pACYCDuet-1, the expression recombinant plasmid pACYC104 was constructed, which allowed the simultaneous expression of genes for the putative acyl-CoA carboxylase α subunit (*nfa9890*; GenBank accession no. YP_117198), the putative acyl-CoA carboxylase β subunit (*nfa9940*; GenBank accession no. YP_117203), the putative biotin-acetyl-CoA carboxylase (ACC)-holoenzyme synthetase (*nfa9950*; GenBank accession no. YP_117204), and the acetyl-CoA synthetase (*nfa3550*; GenBank accession no. YP_116561) from *N. farcinica* IFM10152 (provided by the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan) in *E. coli* to improve intracellular malonyl-CoA levels. Primer pairs *nfa9890* F/*nfa9890* R and *birA* F/*birA* R were used for the amplification of nucleotide sequences of *nfa9890* (1,797 bp) and *nfa9950* (822 bp), respectively, from the genomic DNA of *N. farcinica*. The PCR products of *nfa9890* and *nfa9950* were cloned into the NcoI/BamHI (MCS1) and NdeI/BglII (MCS2) sites of pACYCDuet-1, respectively, to obtain the pACYC102 recombinant expression plasmid. Using the primer pairs *nfa9940* F/*nfa9940* R and *nfa3550* F/*nfa3550* R, the genes *nfa9940* (1,641 bp) and

nfa3550 (1,953 bp) were first cloned into pET24ma(+) at the NdeI/HindIII sites, generating plasmids pET24-*nfa9940* and pET24-*nfa3550*, respectively. Subsequently, using the primer pair 24RBST7 F1/*nfa3550* R1 with pET24-*nfa3550* as a template, PCR was performed, which allowed the amplification of the T7lac sequence, along with the *nfa3550* structural gene. The PCR product, T7-rbs-*nfa3550*, was then cloned into the BglII/MunI sites of pACYC102 to create pACYC103. Likewise, using the primer pair 24RBST7 F2/*nfa9940* R with pET24-*nfa9940* as a template, PCR was performed to amplify the T7lac sequence, along with the *nfa9940* structural gene, which was then cloned into the EcoRI/HindIII sites of pACYC103 to construct the pACYC104 expression recombinant plasmid.

Similarly, the primer pair AT3G51240 F/AT3G51240 R was used for the amplification of the naringenin-3-dioxygenase gene (*AT3G51240*; GenBank accession no. NP_190692) from the cDNA of *A. thaliana*, and the PCR product was cloned into pCDFDuet-1 and pC-*Pc4cl2* in the NcoI/EcoRI sites to construct the pC-*Atf3h* and pC-*Atf3h-Pc4cl2* expression recombinant plasmids, respectively.

To express the O-methyltransferase gene (*SAV_2382*; GenBank accession no. NP_823558), the primers *SaOMT* F and *SaOMT* R were used for amplification of the gene from the genomic DNA of *Streptomyces avermitilis* MA4680 (KCTC, Daejeon, South Korea). Then, the PCR product (1,080 bp) was cloned into pRSFDuet-1 excised with BamHI/EcoRI to construct the pRSF-*Saomt* expression recombinant plasmid. In all cases, construction of the recombinant plasmids was verified by both restriction mapping and direct nucleotide sequencing of the PCR-amplified products of the respective genes in the recombinant plasmids.

Extraction and analysis of intracellular malonyl-CoA. *E. coli* strains BL21 and ACYC104 were grown in 50 ml of M9 minimal medium. To measure dry cell weight (DCW) at different time points, 5 ml of culture

was centrifuged at $10,000 \times g$ for 12 min, and then the supernatant was removed. The cell pellet was washed with distilled water, transferred to a preweighed Eppendorf tube, and dried in an oven. In parallel, 5 ml of culture broth was collected, chilled immediately on ice, and centrifuged at 4°C at $10,000 \times g$ for 5 min, and then the cell pellet was resuspended in 500 μl of medium to extract intracellular malonyl-CoA, as reported previously with some modifications (29). To a 2-ml microcentrifuge, 500 μl of 15% (wt/vol) trichloroacetic acid (TCA) and 800 μl of a silicone oil mixture (AR200-DC200, 2:1; density [d] = 1.01) were added; then, 500 μl cell suspension was poured carefully onto the silicone oil layer without perturbing it. The tube was centrifuged at $10,000 \times g$ for 5 min at 4°C , and 300 μl of the TCA extract (60% of the original cell extract) was passed through an Oasis HLB SPE cartridge under vacuum as follows. The cartridge was first conditioned with 2 ml of methanol, followed by 2 ml of 0.15% TCA solution. The extracts were then applied to the cartridge, followed by 2 ml of 0.15% TCA solution as a washing step. The bound CoA esters were eluted two times with 500 μl of 5% NH_4OH in methanol, evaporated to dryness using a vacuum centrifuge (BioTron, South Korea), and kept in a freezer until analysis. The dried samples were dissolved in 100 μl of water and analyzed by high-performance liquid chromatography (HPLC) (Autochro-3000; Young Lin, South Korea) as described by Fowler et al. (8) at 254 nm.

Recombinant protein expression and flavonoid production and extraction. *E. coli* BL21(DE3) harboring recombinant plasmids was precultured into 3 ml of LB liquid medium with appropriate antibiotics and incubated at 37°C and 200 rpm overnight. The following day, 500 μl of preinoculum was transferred into 20 ml of LB liquid medium (with antibiotics) and cultured at 37°C until the optical density at 600 nm (OD_{600}) reached approximately 0.6. Then, isopropyl- β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and the culture was incubated at 28°C for 20 h. The cells were harvested by centrifugation, washed with 10 ml of phosphate buffer (pH 7.0), and then resuspended in 2 ml of phosphate buffer. The recombinant protein was released by sonication and checked by SDS-PAGE (see Fig. S1 in the supplemental material).

For flavonoid production, after IPTG induction, the culture was incubated at 28°C for 5 h to increase biomass. The cell pellet was collected by centrifugation and resuspended in M9 minimal medium or LB medium (with the same volume as that of culture broth before centrifugation) with 1 mM IPTG. The culture broth was aliquoted (500 μl in each well) in a 96-deep-well plate (Bioneer, Daejeon, Korea) and supplemented with various concentrations of *p*-coumaric acid (50 μM to 500 μM). The plate was then incubated at 28°C and 500 rpm for 24 h. The culture broths were extracted with equal volumes of ethyl acetate (Junsei, Japan). The organic layer was collected and concentrated to dryness by evaporation of excess solvent. The remaining products were dissolved in methanol for HPLC analysis or in ethyl acetate for gas chromatography (GC) analysis.

Flavonoid analysis and quantification. The production of flavonoid(s) in *E. coli* recombinant strains was analyzed and quantified with a high-performance liquid chromatograph equipped with a C_{18} column (4.6 by 150 mm; 5.0- μm particle size; Waters) connected to a UV detector (290 nm). A flow rate of 1 ml/min was used with a linear gradient of water containing 0.1% trifluoroacetic acid buffer (phase A) and acetonitrile (phase B) by the following method: 0 to 2 min (5% B), 2 to 20 min (5 to 50% B), 20 to 24 min (50% B), and 24 to 28 min (50 to 5% B). Under these conditions, the retention time for *p*-coumaric acid is 12.7 min, that for DHK is 15.8 min, that for NRN is 19.7 min, that for 7-OMA is 20.3 min, and that for sakuranetin (SKN) is 24.1 min. For quantification of flavonoids, a calibration curve of authentic NRN was drawn, using 10-, 20-, 30-, 40-, and 50- $\mu\text{g}/\text{ml}$ concentrations.

For GC-mass spectrometry (MS) analysis, the isolated products were converted into their trimethylsilyl (TMS) derivatives by heating at 60°C for 30 min with *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA). GC-MS was carried out on a Finnigan MAT system (gas chromatograph model AG3000 Trace GC Ultra ITQ1100) connected to an ion trap mass

detector. The TMS derivatives were analyzed by using a nonpolar capillary column (5% phenylmethylsiloxane capillary; 30 m by 250- μm inside diameter [i.d.]; 0.25- μm film thicknesses) and a linear temperature gradient (100°C for 1 min, temperature gradient of $30^\circ\text{C min}^{-1}$ to 250°C , hold for 10 min, then 1°C min^{-1} to 275°C , and hold for 2 min). The injector port temperature was 100°C . The scan spectrum was 50 to 650 m/z , and the mass spectrum was obtained by electron impact ionization at 70 eV. Selected ion mode was used for the detection of flavonoids.

Structural elucidation of flavonoids. The recombinant strain *E. coli* F3H-OMT (*E. coli* harboring pC-*Atf3h* and pRSF-*Saomt*) was cultured in 1 liter of LB medium. During induction by IPTG, 250 μM NRN was also added, and after 24 h of incubation, isolation processes were undertaken as described above. The extracted crude compound was chromatographed on Prep-HPLC (Shimadzu, Japan) under the following conditions: column, Alltech Econosil C_{18} 10 U (22 by 250 mm; 5- μm particle size; Alltech Associates, Inc., Deerfield, IL); UV detection, 290 nm; flow rate, 10.0 ml/min; and solvent gradient conditions similar to those mentioned above. The fractions were collected, and the purified fractions were completely dried in a lyophilizer (FDU-830; Eyela, Japan). The structural elucidation of the purified compounds was done by ^1H nuclear magnetic resonance (NMR) spectroscopy.

NMR spectra were obtained in DMSO- d_6 (Aldrich, Chicago, IL) using a Bruker Advance 600 instrument (600 MHz). For the ^1H -NMR experiment, 32 transient spectra were acquired with a spectral width of 8,000 Hz. All NMR data were processed using XWINNMR (Bruker). The structures of NRN, DHK, SKN, and 7-OMA were determined based on the interpretation of the NMR data (9, 45).

RESULTS

Improvement of intracellular malonyl-CoA. In flavonoid biosynthesis, the key step is the condensation of three molecules of malonyl-CoA with a molecule of *p*-coumaroyl-CoA to generate the C_{15} chalcone intermediate (19, 27). Since a low concentration of the intracellular malonyl-CoA pool is a major barrier for large-scale production of flavonoids, we undertook the improvement of the intracellular malonyl-CoA concentration by overexpressing the ACC α and β subunits, biotin-ACC-holoenzyme synthetase, and acetyl-CoA synthetase from *N. farcinica*. Although they belong to the actinobacteria, as *Streptomyces* strains also do, most of the proteins from *Nocardia* strains were well expressed in *E. coli* (unpublished data), which is the reason we chose the corresponding genes from *N. farcinica*. All four of the genes were cloned into a single vector, pACYCDuet-1 (low copy number, 10 copies) through subcloning, where the expression of each gene was regulated individually by the *T7lac* promoter. The constructed recombinant plasmid pACYC104 was transformed into *E. coli* BL21(DE3), and the level of intracellular malonyl-CoA was analyzed. In the recombinant strain *E. coli* ACYC104, the concentration of malonyl-CoA was increased about 2.3-fold, i.e., from 0.88 to 2.01 nmol/mg DCW at 6 h of the growth phase (Fig. 2).

Biosynthesis of naringenin and malonyl-CoA overproduction. The recombinant plasmids pC-*Pc4cl2* and pE-*Phchs-Mschi* were transformed into *E. coli* BL21(DE3) to construct *E. coli* strain E2 (19) synthesizing NRN. This strain produced 5.5 mg/liter (20.2 μM) NRN upon being fed 500 μM *p*-coumaric acid in LB medium. The NRN production by the strain was limited due to an inadequate supply of intracellular malonyl-CoA. To enhance its production, the recombinant plasmid pACYC104 was introduced into *E. coli* strain E2, resulting in *E. coli* strain E3. Although the pACYC104 plasmid effectively increased the malonyl-CoA pool in *E. coli*, we could not obtain noticeable enhancement of NRN production by strain E3 in LB medium (see Fig. 4a), i.e., only 6.5

FIG 2 Comparison of intracellular malonyl-CoA production by *E. coli* strains BL21 and ACYC104.

mg/liter (23.8 μ M) of NRN was produced by strain E3 in LB medium at a 500 μ M substrate concentration. For further production improvement, carbon and nitrogen sources were optimized by analyzing NRN production in strain E3 using minimal medium containing different carbon (1%) and nitrogen (0.1%) sources supplementing 500 μ M *p*-coumaric acid. Among the carbon sources tested, glucose was the most effective for NRN production, whereas variation in nitrogen sources was found to have negligible effects (data not shown). Further, the production of NRN by strains E2 and E3 was assessed at various substrate concentrations ranging from 50 to 500 μ M in M9 minimal medium containing 1% glucose and 0.1% NH_4Cl (as described in Materials and Methods). Using a 250 μ M initial *p*-coumaric acid concentration, strains E2 and E3 yielded 6.1 mg/liter (22.4 μ M) and 13.5 mg/liter (49.5 μ M) NRN at 24 h, respectively (see Fig. 4a). Hence, a 2.2-fold increase in NRN production was achieved by strain E3 in M9 minimal medium at a 250 μ M substrate concentration. The production of NRN was verified by comparison with authentic NRN in HPLC (Fig. 3) and also by GC-MS analysis after TMS derivatization, which gives m/z 473 for NRN (see Fig. 6a).

Production of dihydrokaempferol. To introduce a hydroxyl group at the C-3 position of NRN, the recombinant plasmid pC-*Atf3h-Pc4cl2* was constructed by cloning the *AT3G51240* gene, encoding naringenin-3-dioxygenase (flavonone-3-hydroxylase), from *A. thaliana* into pC-*Pc4cl2*. The constructed recombinant plasmid, along with pE-*Phchs-Mschi* and pACYC104, was transformed into *E. coli* BL21(DE3) to generate the *E. coli* E3FH recombinant strain, which is capable of producing DHK from *p*-coumaric acid. The production of DHK by strain E3FH was analyzed at various *p*-coumaric acid concentrations in both LB and M9 minimal media at 24 h. In LB medium at 500 μ M *p*-coumaric acid, strain E3FH produced 9.7 mg/liter (33.6 μ M) DHK, whereas 16.5 mg/liter (57.2 μ M) DHK was produced in M9 minimal medium at a 250 μ M substrate concentration (Fig. 4b). The NRN produced from *p*-coumaric acid was completely converted to DHK in the presence of the *AT3G51240* gene, resulting in the absence of NRN in the culture broth (Fig. 3d). The production of DHK was detected by HPLC with a peak showing a retention time of 15.8 min, and it was further confirmed by GC-MS analysis, which gives an m/z ratio of 561 for TMS-derivatized

FIG 3 HPLC profile of standard naringenin (a), *p*-coumaric acid-supplemented *E. coli* strain E2 in M9 medium (b), *E. coli* strain E3 in M9 medium (c), *E. coli* E3FH in M9 medium (d), *E. coli* E3MT in LB medium (e), and *E. coli* E3FHT in LB medium (f). ∇ , *p*-coumaric acid peak; $\bullet$, NRN peak; $\star$, DHK peak; $\circ$, SKN peak; $\ast$, 7-OMA peak.

DHK (see Fig. 6b) and $^1\text{H-NMR}$ spectroscopy (see Fig. S3b in the supplemental material).

Production of DHK was also analyzed in an *E. coli* F3H (*E. coli* BL21 harboring the pC-*Atf3h* recombinant plasmid) strain supplemented with various concentrations of NRN in LB medium. This strain produced ca. 71.3 mg/liter (247.3 μ M) DHK at 24 h upon feeding 500 μ M NRN, i.e., 49.5% conversion of NRN into DHK (Fig. 5a).

Production of sakuranetin. To carry out *O*-methylation at the C-7 position of flavonoid, the *SaOMT* gene encoding 7-*O*-methyltransferase from *S. avermitilis* was cloned into the pRSFDuet-1 expression vector to construct the pRSF-*Saomt* expression plasmid. The recombinant plasmids pC-*Pc4cl2*, pE-*Phchs-Mschi*, pRSF-*Saomt*, and pACYC104 were transformed into *E. coli* BL21(DE3) to construct *E. coli* strain E3MT. The strain was applied to produce methylated derivatives of SKN from *p*-coumaric acid. Despite production of 11.3 mg/liter (41.5 μ M) NRN, only 382 μ g/liter (1.3 μ M) SKN was produced in M9 minimal medium at 500 μ M *p*-coumaric acid. Similarly, in LB medium, 517 μ g/liter (1.8 μ M) SKN and 6.5 mg/liter (23.8 μ M) NRN were produced by strain E3MT at 100 μ M *p*-coumaric acid (Fig. 4c). The SKN production from *E. coli* strain OMT (*E. coli* BL21 harboring plasmid pRSF-*Saomt*) was also analyzed by supplementing various concentrations of NRN in LB medium. Upon feeding 500 μ M NRN, *E. coli* OMT produced 43.2 mg/liter (150.9 μ M) SKN with more than 50% of the substrate remaining unreacted in the medium (Fig. 5b). In HPLC, SKN was detected with a retention time of 24.1 min, and it was further confirmed by GC-MS analysis, which gives an m/z of 415 for TMS-derivatized

FIG 4 Profiles of flavonoid production by *E. coli* recombinant strains upon supplementation with various concentrations of *p*-coumaric acid in M9 minimal and LB media. (a) NRN production by *E. coli* strains E2 and E3 at 24 h. (b) DHK production by *E. coli* strain E3FH at 24 h. (c) NRN and SKN production by *E. coli* strain E3MT at 24 h. (d) NRN, 7-OMA, DHK, and SKN production by *E. coli* strain E3FHMT at 24 h.

SKN (Fig. 6c) and $^1\text{H-NMR}$ spectroscopy (see Fig. S3c in the supplemental material).

Production of 7-O-methyl aromadendrin. For the production of 7-OMA, an *E. coli* BL21 derivative strain harboring pC-

Atf3h-Pc4cl2, pE-*Phchs-Mschi*, pRSF-*Saomt*, and pACYC104 recombinant plasmids (*E. coli* strain E3FHMT) was constructed. This strain produced 3.2 mg/liter (11.1 μM) DHK, 2.7 mg/liter (8.9 μM) 7-OMA, and 185 $\mu\text{g/liter}$ (0.6 μM) SKN in LB medium

FIG 5 Profiles of flavonoid production by *E. coli* recombinant strains upon supplementation with various NRN concentrations in LB medium. (a) DHK production by *E. coli* strain F3H at 24 h. (b) SKN production by *E. coli* strain OMT at 24 h. (c) 7-OMA, DHK, and SKN production by *E. coli* strain F3H-OMT at 24 h.

using 500 μM *p*-coumaric acid, whereas 3.8 mg/liter (13.18 μM) DHK and 1.2 mg/liter (3.9 μM) 7-OMA were produced in M9 minimal medium from 250 μM *p*-coumaric acid in the medium (Fig. 4d).

For higher production of 7-OMA starting from NRN, the recombinant plasmids pC-*Atf3h* and pRSF-*Saomt* were transformed into *E. coli* BL21 to construct *E. coli* F3H-OMT. At 500 μM NRN, 63.5 mg/liter (220.3 μM) DHK, 30.0 mg/liter (99.2 μM) 7-OMA, and 12.7 mg/liter (44.3 μM) SKN were produced in LB medium using the strain (Fig. 5c). The production of 7-OMA was detected at 20.3 min in HPLC, which was further confirmed by GC-MS with *m/z* 503 for TMS-derivatized 7-OMA (Fig. 6d) and ¹H-NMR analysis (see Fig. S3d in the supplemental material).

During the biosynthesis of 7-OMA, hydroxylation of NRN at the C-3 position could be followed by *O*-methylation at the C-7 position (pathway a in Fig. 1); *O*-methylation might occur first,

followed by hydroxylation (pathway b in Fig. 1); or simultaneous actions of both pathways might be possible. To determine the exact pathway, conversion of DHK by *E. coli* strain OMT and conversion of SKN into 7-OMA by *E. coli* F3H were analyzed. In both cases, formation of 7-OMA was shown by the corresponding peak in the HPLC profile at 20.3 min (Fig. 7). Although both pathways are possible, a larger amount of 7-OMA is produced from the conversion of DHK than from SKN, suggesting that pathway a in Fig. 1 is the dominant pathway.

Structural elucidation of NRN, DHK, SKN, and 7-OMA. In the full GC-MS spectrum, TMS derivatives of flavonoids (*M*⁺ molecular ions), ions with the loss of one methyl group (*[M-CH₃]*⁺), and other fragment ions formed via retro-Diels-Alder (rDA) reaction at the A, B, and C rings were observed (Fig. 6). The mass spectra of the trimethylsilylated *[M-CH₃]*⁺ ion of NRN, DHK, SKN, and 7-OMA are characterized by mass peaks at 473, 561, 415, and 503, respectively. Since all four flavonoids have the same B-ring structure, they gave a common ion mass peak of *m/z* 179. The C-ring cleavage of NRN and DHK produced the mass peak of diene (A ring) at *m/z* 369, but due to the presence of an OCH₃ group at the C-7 position in SKN and 7-OMA, the mass peak of diene appears at *m/z* 311. Similarly, C-ring cleavage of NRN and SKN produced mass spectra (B ring) of *m/z* 192, whereas the corresponding fragments for DHK and 7-OMA appear at *m/z* 280 because of the OH group at the C-3 position (see Fig. S2 in the supplemental material). Furthermore, the structures of DHK, SKN, and 7-OMA were confirmed by ¹H-NMR analysis (Table 2; see Fig. S3 in the supplemental material). The ¹H-NMR signals of DHK at δ 5.04 (1H, d, *J* = 11.4 Hz, H-2) and δ 4.58 (1H, d, *J* = 11.4 Hz, H-3) showed only one proton at the C-3 position, confirming the OH group attached to this carbon. The presence of the OCH₃ group of SKN in the C-7 position was shown by the ¹H-NMR signal at δ 3.78 (3H, s). In the case of 7-OMA, the ¹H-NMR signal at δ 5.10 (1H, d, *J* = 11.52 Hz, H-2) and δ 4.64 (1H, d, *J* = 11.52 Hz, H-3) confirmed the OH group attached to C-3, and the OCH₃ group at the C-7 position was shown by δ 3.78 (3H, s).

DISCUSSION

Flavonoids are widely used in the food-processing, pharmaceutical, and cosmetic industries. There is increasing interest in developing, and need to develop, alternative sources for the production and development of flavonoid analogues. Since not only has the flavonoid biosynthetic pathway been almost completely elucidated (7), but next-generation sequencing technology can also accelerate genome sequencing of rare medicinal herbs and plants, combinatorial biosynthesis of the flavonoids in recombinant *E. coli* or yeast has drawn significant attention. The functional-group substitution of flavonoids can lead to the generation of compounds with improved pharmacological properties. For example, hydroxylation and/or glycosylation of flavonoid compounds has a profound impact on their solubility, stability, biological activity, and detoxification properties (38, 40). Similarly, methylated flavonoids exhibited superior anticancer activity compared to the corresponding hydroxylated derivatives, becoming more resistant to the hepatic metabolism and showing higher intestinal absorption (41). 7-OMA possesses a hydroxyl group at the C-3 position and an *O*-methyl group at the C-7 position compared to NRN. It showed significantly enhanced insulin-stimulated glucose uptake compared to sakuranetin and rosiglitazone (an antidiabetic drug) (44). Several side effects, such as upper respiratory tract infection,

FIG 6 GC-MS profiles of NRN from *E. coli* strain E3 (a), DHK from *E. coli* strain E3FH (b), SKN from *E. coli* strain E3MT (c), and 7-OMA from *E. coli* strain E3FHMT (d).

FIG 7 HPLC profiles of standard DHK, 7-OMA, and SKN (a); DHK-supplemented *E. coli* strain OMT in LB medium (b); and SKN-supplemented *E. coli* strain F3H in LB medium (c). ★, DHK peak; *, 7-OMA peak; ○, SKN peak.

chest and back pain, bone fractures, macular edema, and heart failure, have been associated with the drug rosiglitazone (14, 23, 34). In addition, daily doses of rosiglitazone range from 4 to 8 mg (32), and there is no additional benefit to doses greater than 8

mg/day. In contrast, 7-OMA, as a flavonoid compound, could be taken up abundantly without such side effects, so it could be a potential candidate for the development of antidiabetic drugs in the future. Due to these beneficial properties, we were interested in its efficient production.

A slower growth profile, difficulties in harvesting sufficient amounts of the desired plants, varying compositions of compounds depending upon geographical and climatic conditions, low yield with unnecessary background metabolites due to a large genome size, and complexity in genetic manipulation are the major hurdles for industrial-scale flavonoid production by their native producers (16). Chemical synthesis of flavonoids is often complex and requires toxic chemicals and extreme reaction conditions (37). In addition, chiral synthesis to form active flavonoid molecules and other modifications, such as glycosylation, are crucial challenges in chemical synthesis. On the other hand, the Gram-negative bacterium *E. coli* has become one of the most promising microbial hosts, with a highly tractable genetic system and favorable fermentation conditions for production purposes (8, 20). In addition, the degree of complexity in *E. coli* is significantly lower than that of plants due to its smaller genome. Several studies showed that high productivities and yields of flavonoids can be achieved from recombinant *E. coli* strains. For example, heterologous expression of phenylalanine ammonia lyase (PAL) from the yeast *Rhodotorula rubra*, of 4CL from the actinomycete *Streptomyces coelicolor* A3 (2), and of CHS from the licorice plant *Glycyrrhiza echinata* in *E. coli* BL21 resulted in the production of 452.6 $\mu\text{g/liter}$ NRN upon supplementation with 2 mM tyrosine (13). Furthermore, incorporation of CHI from the *Pueraria* plant and overexpression of two subunits of ACC from *Corynebacterium glutamicum* in the recombinant *E. coli* strain improved the production of NRN to 60 mg/liter in the presence of 3 mM tyrosine (27). Similarly, Leonard and coworkers obtained 119 mg/liter NRN from 3 mM *p*-coumaric acid by heterologous expres-

TABLE 2 $^1\text{H-NMR}$ of NRN, DHK, SKN, and 7-OMA^a

Position of H	$^1\text{H-NMR}$ analysis			
	NRN	DHK	SKN	7-OMA
2	5.44, dd ($J = 2.88, 12.84$ Hz)	5.04, d ($J = 11.4$ Hz)	5.48, dd ($J = 2.82, 12.84$)	5.10, d ($J = 11.52$ Hz)
3 eq	2.68, cis, dd ($J = 3, 17.1$ Hz)	4.58, d ($J = 11.4$ Hz)	2.72, dd ($J = 2.94, 17.1$ Hz)	4.64, d ($J = 11.52$ Hz)
3 axial	3.26, trans, q ($J = 12.84, 17.1$ Hz)		3.32, trans, q ($J = 9.24, 13.44$ Hz)	
4				
5				
6	5.87, s	5.85, s ($J = 1.8$ Hz)	6.09, dd ($J = 2.28, 14.52$ Hz)	6.10, q ($J = 2.22, 13.62$ Hz)
7				
8	5.87, s	5.91, s ($J = 1.86$ Hz)	6.09, dd ($J = 2.28, 14.52$ Hz)	6.10, q ($J = 2.22, 13.62$ Hz)
9				
10				
1'				
2'	7.31, d ($J = 8.52$ Hz)	7.30, d ($J = 8.4$ Hz)	7.32, d ($J = 8.58$ Hz)	7.32, d ($J = 8.52$ Hz)
3'	6.79, d ($J = 8.52$ Hz)	6.78, d ($J = 8.4$ Hz)	6.79, d ($J = 8.52$ Hz)	6.78, d ($J = 8.52$ Hz)
4'				
5'	6.79, d ($J = 8.52$ Hz)	6.78, d ($J = 8.4$ Hz)	6.79, d ($J = 8.52$ Hz)	6.78, d ($J = 8.52$ Hz)
6'	7.31, d ($J = 8.52$ Hz)	7.30, d ($J = 8.4$ Hz)	7.32, d ($J = 8.58$ Hz)	7.32, d ($J = 8.52$ Hz)
5-OH	12.14, s	11.90, s	12.11, s	11.87, s
7-OH	10.77, s	10.81, s		
4'-OH	9.57, s	9.54, br s	9.59, s	9.56, s
7-OCH ₃			3.78, s	3.78, s

^a At 600 MHz in DMSO- d_6 .

sion of 4CL from *Petroselinum crispum*, CHS from *Petunia hybrida*, and CHI from *Medicago sativa* in the presence of four ACC subunits and biotin ligase from *Photorhabdus luminescens*, along with *E. coli* genes from the acetate assimilation pathway (19). On the other hand, simultaneous expression of a malonate assimilation pathway, i.e., expression of malonate synthetase (*matB*) and malonate carrier protein (*matC*) from *Rhizobium trifolii* with the flavanone biosynthetic 4CL, CHS, and CHI genes from the same plants mentioned above resulted in the production of 155 mg/liter NRN (20). Likewise, production of the flavones apigenin (415 μ g/liter), luteolin (10 μ g/liter), and genkwanin (208 μ g/liter) from a recombinant *E. coli* strain expressing 4CL and of flavone synthase from parsley, CHS and CHI from *P. hybrida*, and 7-O-methyltransferase from peppermint was achieved (18).

E. coli generates very low levels of intracellular malonyl-CoA (36), which is a potential barrier to wide utilization of this host for commercial-scale production of flavonoids and other important polyketides. In the present study, the intracellular malonyl-CoA pathway was engineered by overexpressing ACC and ACS genes from *N. farcinica*, which resulted in a 2.3-fold increase of the malonyl-CoA levels. Incorporation of the flavonoid biosynthesis pathway into this malonyl-CoA engineered strain and optimization of the medium composition resulted in a 2.2-fold increase in the production of NRN upon feeding with 250 μ M *p*-coumaric acid. This increase in NRN production by *E. coli* strain E3 using *p*-coumaric acid was observed only in minimal medium with glucose as a carbon source, but not in LB medium. Since the larger amount of *p*-coumaric acid was more rapidly degraded by *E. coli* in LB medium than in M9 minimal medium (see Fig. S4 in the supplemental material), the prominent substrate degradation might be the major cause for the lower yield of NRN production in LB medium. On the other hand, glucose was the best carbon source for flavonoid production, since it is effective in increasing the intracellular acetyl-CoA concentration via the glycolysis pathway (36).

In order to produce DHK from NRN, we initially chose two oxidoreductases belonging to the 2-oxoglutarate and Fe(II) oxygenase families from *A. thaliana*: naringenin-3-dioxygenase with flavanone 3-hydroxylase activity (*AT3G51240*) and oxidoreductase (*AT5G20400*; GenBank accession no. NP_197540), which is similar to flavanone 3-hydroxylase from *Malus domestica*. The conversion of NRN to DHK was achieved in the presence of *AT3G51240*, but not with *AT5G20400*. On the other hand, it was reported that 7-O-methyltransferase from *S. avermitilis* (*SaOMT*) can efficiently convert isoflavanones, flavones, and flavanones, including NRN, into the corresponding 7-O-methylated derivatives (15). For this reason, we used the enzyme for further conversion of DHK into 7-OMA. Starting from *p*-coumaric acid, only 3.8 mg/liter DHK, 2.7 mg/liter 7-OMA, and trace amounts (185 μ g/liter) of SKN were produced by *E. coli* strain E3FHMT. To yield higher production of these flavonoids, *E. coli* strain F3H-OMT was constructed. Upon feeding 500 μ M NRN, 63.5 mg/liter DKH (44% conversion), 30.0 mg/liter 7-OMA (19.8% conversion), and 12.7 mg/liter SKN (8.9% conversion) were obtained from the strain.

E. coli strain E3FH completely converted the biosynthesized NRN from *p*-coumaric acid into DHK (Fig. 4b), whereas SKN was produced in *E. coli* E3MT only on a microgram scale, leaving a large amount of NRN unused (Fig. 4c). In addition, the conversion of NRN into DHK by F3H is higher than the conversion of

NRN into SKN by *SaOMT* (Fig. 5a and b). Thus, it can be anticipated that after the formation of NRN from *p*-coumaric acid in *E. coli* E3FHMT, a larger fraction of NRN was converted to DHK. From the intermediate compound (i.e., DHK or SKN) feeding experiment (Fig. 7), it was found that a larger amount of 7-OMA was produced from DHK by the action of *SaOMT* than from SKN by the action of F3H. Considering these factors, our findings demonstrate that although both pathways (a and b) can produce 7-OMA, it is very likely that the major pathway constitutes hydroxylation at the C-3 position, followed by *O*-methylation at the C-7 position.

Although we generated 7-OMA starting from *p*-coumaric acid using *E. coli*, the production level of the final compound is very low compared to the consumption of *p*-coumaric acid. Figure 3e and f show that almost all *p*-coumaric acid was consumed by the recombinant strains in LB medium, suggesting that its degradation pathway is more pronounced under this growth condition. Previously, it was reported that *E. coli* strains can use several aromatic acids as sole carbon sources for their growth (3, 4, 5). We found that almost 40 to 60% of *p*-coumaric acid was degraded by *E. coli* BL21(DE3) in M9 minimal medium, whereas almost 98% degradation was observed in LB medium at 24 h (see Fig. S4 in the supplemental material). Identical degradation pathways of 4-hydroxyphenylacetate (4) and 3-phenylpropionic acid (3) in *E. coli* have been reported, which are also responsible for the catabolism of cinnamic acid and its derivatives (5). Inactivation of these pathways can lead to stability of aromatic acids, including *p*-coumaric acid, in *E. coli* and hence possibly improves the yields in the production of flavonoids.

In conclusion, we have successfully generated medicinally important plant-based flavonoids in *E. coli* using a combinatorial approach. The compound DHK is one of the precursor metabolites for flavonol, anthocyanidin, proanthocyanidin, and catechin biosynthesis. Since the production of SKN was very low in *E. coli* E3MT, its production could be enhanced by expressing other 7-O-methyltransferases with higher catalytic activities. Similarly, the activity of 7-OMA can be increased by modifications, such as glycosylation, acetylation, or methylation, at the C-3 position. Future efforts need to improve production of the above-mentioned compounds by overcoming the bottleneck steps during biotransformation.

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