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Strain Improvement of Recombinant *Escherichia coli* for Efficient Production of Plant Flavonoids

Effendi Leonard, Yajun Yan, Zachary L. Fowler, Zhen Li, Chin-Giaw Lim, Kok-Hong Lim, and Mattheos A. G. Koffas*

Department of Chemical and Biological Engineering, University at Buffalo, Buffalo, New York 14260

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Abstract: Plant flavonoid polyphenols continue to find increasing pharmaceutical and nutraceutical applications; however their isolation, especially of pure compounds, from plant material remains an underlying challenge. In the past *Escherichia coli*, one of the most well-characterized microorganisms, has been utilized as a recombinant host for protein expression and heterologous biosynthesis of small molecules. However, in many cases the expressed protein activities and biosynthetic efficiency are greatly limited by the host cellular properties, such as precursor and cofactor availability and protein or product tolerance. In the present work, we developed *E. coli* strains capable of high-level flavonoid synthesis through traditional metabolic engineering techniques. In addition to grafting the plant biosynthetic pathways, the methods included engineering of an alternative carbon assimilation pathway and the inhibition of competitive reaction pathways in order to increase intracellular flavonoid backbone precursors and cofactors. With this strategy, we report the production of plant-specific flavanones up to 700 mg/L and anthocyanins up to 113 mg/L from phenylpropanoic acid and flavan-3-ol precursors, respectively. These results demonstrated the efficient and scalable production of plant flavonoids from *E. coli* for pharmaceutical and nutraceutical applications.

Keywords: Flavonoids; anthocyanins; malonyl-CoA; UDP-glucose; *E. coli*

Introduction

With over 9000 substituted members and growing, flavonoids are among the most numerous and widespread plant secondary metabolites[1] found in fruits, vegetables, nuts, seeds, herbs, spices, stems, flowers, teas and red wines. The core flavonoid structure is composed of two benzene rings which are linked by a heterocyclic pyran or pyrone ring. The variability in molecular structure subdivides flavonoids into flavonols, flavones, flavanones, isoflavones, catechins and anthocyanins. Chemical diversification is the result of an assortment of chemical modifications, such as methylations, methoxylations, acylations, *C*- and *O*- glycosylations and hydroxylations.

Flavonoids have been associated with the health benefits of diets rich in fruits and vegetables and may be at least partially responsible for the so-called French paradox, the reduction in coronary artery disease among Mediterranean people with fat-rich diets that include significant amount of red wine.[2] Accumulating evidence points to the beneficial effects of flavonoids in human chronic conditions, such as diabetes and obesity as they can cause insulin-like effects by reducing blood glucose levels after food intake.[3,4]

* To whom correspondence should be addressed. Mailing address: University at Buffalo, SUNY, Chemical and Biological Engineering, 904 Furnas Hall, North Campus, Buffalo, NY 14260. E-mail: mkoffas@eng.buffalo.edu. Phone: 716-645-2911. Fax: 716-645-3822.

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Previous studies demonstrated that inclusion of anthocyanins in high-fat diets in mice resulted in body weights comparable to those of mice fed with a normal diet. On the other hand, without anthocyanin intake, a high-fat diet resulted in a 25% increase in the mice's body weight. Supporting this finding, the serum glucose and insulin concentrations in mice increased up to 70% under a high-fat diet. However, suppression of glucose and insulin levels was observed when anthocyanins accompanied the high-fat diet.[5,6] Further studies on cellular levels showed that flavonoid treatments in mice enhanced adipocytokine (adiponectin and leptin) secretion and up-regulated adipocyte-specific and adiponectin gene expression in adipocytes and white adipose tissue, respectively.[7] In addition, pure anthocyanins and anthocyanidins have been shown to stimulate insulin secretion from rodent pancreatic beta-cells *in vitro*,[8] similar to the recently reported effect of flavan-3-ols in human pancreatic beta-cells *in vitro*.[4] Polyphenols are also bioactive molecules within the gastrointestinal (GI) tract[9] and can act as inhibitors to starch degradation enzymes, such as α-glucosidase and α-amylase.[10–12] In many cases, the inhibitory effects of the plant polyphenols were shown to be comparable or higher than the currently prescribed drugs, such as acarbose.[12–14]

Despite their numerous health benefits, the high-level production of pure flavonoid compounds from plant extraction continues to be a major challenge for drug development. While chemical syntheses are available for some compounds, they typically involve toxic chemicals and extreme reaction conditions that can make the whole process difficult to scale. Essential modifications, such as glycosylations and chiral synthesis to form active flavonoid molecules, are also crucial challenges in chemical syntheses.[15,16] As a result, fermentation-based flavonoid production is an attractive approach as it has several advantages including the utilization of environmentally friendly feedstocks, low energy requirements and low waste emissions.[17] The generation of flavonoid producer strains by grafting plant biosynthetic pathways has been reported in the past.[18–23] However, low productivity remains a barrier for large-scale production. Recently, it was demonstrated that flavanone production could be elevated up to approximately 400 mg/L[24] while anthocyanin production reached 70 mg/L[25] through the overexpression of rate-limiting enzymes in *E. coli*. In the present work, the

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Table 1. Strains and Plasmids Used in This Work

plasmids	relevant characteristics	source
pETDuet-1	double T7 promotors, ColE1 ori, Amp^R	Novagen
pET-CHI-CHS	pETDuet-1 carrying *M. stavia* CHI and petunia CHS	ref 24
pEGP	pETDuet-1 carrying *E. coli galU* and *pgm*	ref 25
pRSFDuet-1	double T7 promotors, ColDF13 ori, Sm^R	Novagen
pRSF-4CL-FSI	pRSFDuet-1 carrying parsley 4CL and FSI	ref 26
pCDFDuet-1	double T7 promotors, ColDF13 ori, Sm^R	Novagen
pCDF-4CL	pCDFDeut-1 carrying Parsley 4CL	ref 24
pCDF-3A	pCDFDuet-1 carrying *Arabidopsis 3GT* fused to N-terminus of petunia *ANS*	ref 25
pCoLADuet-1	double T7 promotors, ColA ori, Kan^R	Novagen
pCoLA-ndk	pCoLADuet-1 carrying *E.coli ndk*	this study
pCoLA-cmk	pCoLADuet-1 carrying *E.coli cmk*	this study
pCoLA-ndk-cmk	pCoLADuet-1 carrying *E. coli ndk* and *cmk*	this study
pACYCDuet-1	double T7 promoters, P15A ori, Cm^R	Novagen
pACYC-MatB-MatC	pACYCDuet-1 carrying *R. trifolii matB* and *matC*	this study
pACYC-pyrE	pACYCDuet-1 carrying *E. coli pyre*	this study
pACYC-pyrE-pyrF	pACYCDuet-1 carrying *E. coli pyre and pyrF*	this study
strains	description	source
E. coli Top10F′	F′ {lacIq Tn10 (TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
E. coli BL21Star	F- ompT hsdSB (rB-mB-) gal dcm rne131	Invitrogen
E. coli JM109(DE3)	endA1, recA1, gyrA96, thi, hsdR17 (rk−, mk+), relA1, supE44, λ-, Δ(lac-proAB), [F′, traD36, proAB, lacIqZΔM15], lDE3	Promega
E2	*E. coli* BL21Star + pET-CHI-CHS + pCDF-4CL	ref 24
E2M	E2 + pACYC-MatB-MatC	this study
E2V	*E. coli* BL21Star + pET-CHI-CHS + pRSF-4CL-FSI	ref 24
JA	*E. coli* JM109(DE3) + p3A	this study
YA	*E. coli* BL21Star + p3A	ref 25
YAP	YA + pEGP	ref 25
YAN	YAP + pCoLA-ndk	this study
YAC	YAP + pCoLA-ndk-cmk	this study
YAY	YAC + pACYC-pyrE-pyrF	this study
AC	YA + pCoLA-cmk	this study
AP	YA + pACYC-pyrE-pyrF	this study
AY	YA + pACYC-pyrE-pyrF + pCoLA-cmk	this study
YU	*E. coli* BL21Star Δ*udg* + p3A	this study
YUP	YU + pEGP	this study
YUN	YUP + pCoLA-ndk	this study
YUC	YUP + pCoLA-ndk-cmk	this study
YUY	YUPC + pACYC-pyrE-pyrF	this study

metabolism of *E. coli* was diverted through the introduction of novel carbon assimilation pathways and the attenuation of several gene targets in order to increase the availability of malonyl-CoA and UDP-glucose, the rate-limiting metabolites for flavanone and anthocyanin synthesis, respectively. With these genetic and metabolic engineering strategies, the recombinant production of flavanones and anthocyanins reached up to 700 mg/L and 113 mg/L, respectively.

Experimental Details

Chemicals and Bacterial Strains. Luria Broth (LB), Terrific Broth (TB), and M9 minimal media (1x M9 salts, 1% glucose, 6 nM thiamine, 1 μM $MgSO_4$) were used throughout. Various combinations of ampicillin (70 μg/mL), kanamycin (40 μg/mL), chloramphenicol (20 μg/mL), and streptomycin (40 μg/mL) were added to cultures of plasmid-bearing *E. coli* strains. Cinnamic acid, *p*-coumaric acid, and caffeic acid were purchased from MP Biomedicals Inc. *trans*-4-Amino cinnamic, *trans*-2-cinnamic, and *trans*-4-fluorocinnamic acids and (+)-catechin were purchased from TCI America. *trans*-3-Cinnamic acid was purchased from Indofine. *trans*-2-Fluorocinnamic acid was purchased from Alfa-Aesar. Natural flavanone and flavone standards were purchased from Indofine. Anthocyanidin standards were purchased from Extrasynthase while anthocyanin 3-*O*-glucoside standards were purchase from ChromaDex. The compatible vectors pETDuet-1, pCDFDuet-1, pCOLADuet-1 and pACYCDuet-1 were purchased from Novagen. Plasmids pKD4,

Table 2. Oligo Nucleotide Primers Used in This Work

name	sequence (5′−3′)[a]
	For Malonate Assimilation
Pf_RtMatB(*Nde*I)	GGGAAATTTCATATGGCG**ATG**AGCAACCATCTTTTCGACGC
Pr_RtMatB(*Kpn*I)	CCCGGTACCCCC*TTA*CGTCCTGGTATAAAGATCGGC
Pf_RtMatC(*Eco*RI)	CCCGAATTCGGG**ATG**GGTATTGAATTACTGTCCATAGG
Pr_RtMatC(*Hin*dIII)	TTGGGAAGCTTGGG*TCA*AACCAGCCCGGGCACGACG
	For UDP-Glucose Assimilation
ndk-Beg(*Bam*HI)	GGGAAAGGATCC**ATG**GCTATTGAACGTACTTTTTCCATCATCAAACC
ndk-End(*Hin*dIII)	CCCTTTAAGCTT*TTA*ACGGGTGCGCGGGCACACTTCGCCTT
cmk-Beg(*Bgl*II)	GGGAAAAGATCT**ATG**ACGGCAATTGCCCCGGTTATTA
cmk-End(*Xho*I)	CCCTTTCTCGAG*TTA*TGCGAGAGCCAATTTCTGGCGC
pyrE-Beg(*Bgl*II)	GGGAAAAGATCT**ATG**AAACCATATCAGCGCCAGTTTATTGAATTTGC
pyrE-End(*Xho*I)	CCCTTTCTCGAG*TTA*AACGCCAAACTCTTCGCGATAGGCC
pyrF-Beg(*Sac*I)	GGGAAAGAGCTC**ATG**ACGTTAACTGCTTCATCTTCTTCCCGCGC
pyrF-End(*Hin*dIII)	CCCTTTAAGCTT*TCA*TGCACTCCGCTGTAAAGAGGCGTT
UDGCDS-H1P1	**ATG**AAAATCACCATTTCCGGTACTGGCTATGTAGGCTTGTGTGTAGGCTGGAGCTGCTTC
UDGCDS-P2H2	*TTA*GTCGCTGCCAAAGAGATCGCGGGTGTATACCTTATCTATGGGAATTAGCCATGGTCC
UDG-Check-Beg	*TTA*GTCGCTGCCAAAGAGATCGCGG
UDG-Check-End	**ATG**AAAATCACCATTTCCGGTACTGGC

[a] Underlining indicates restriction enzyme cleavage sites corresponding to the primer description for cloning primers or homologous extensions for deletion primers. Boldface indicates start codon, and italics indicate stop codon.

pKD46 and pCP20 were acquired from Yale *E. coli* Genetic Stock Center and were used for gene disruption. All restriction enzymes and DNA ligase were purchased from New England Biolabs. QIAGEN RNeasy MiniKit was used for total RNA isolation. Genomic DNA was isolated using the PureLink Genomic DNA Isolation Kit (Invitrogen). cDNAs were obtained using Superscript One-step with Platinum Taq kit (Invitrogen). Gene amplification utilized Accuzyme DNA polymerase (BioLine). *E. coli* TOP10F′ (Invitrogen) was used for plasmid propagation while JM109(DE3) and BL21Star (Invitrogen) were used for recombinant production. *R. trifolii* was purchased from ATCC. All strains constructed and used in the study can be found in Table 1, and oligonucleotide primers used for cloning and PCR can be found in Table 2.

Plasmid and Recombinant Strain Constructions. For *R. trifolii* RNA isolation, cells were grown in liquid ATCC medium 111 with 1 g/L malonate supplementation. *R. trifolii matB* and *matC* genes encoding for malonate synthetase and malonate carrier protein, respectively, were isolated by RT-PCR and were individually cloned into vector pACYCDuet-1 in between *Nde*I and *Kpn*I, and *Eco*RI and *Hin*dIII sites, respectively, creating plasmid pACYC-MatB-MatC. The construction of flavanone producing *E. coli*, E2, which expressed plant-specific 4-coumaroyl:CoA ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI), was previously described.[24] To create the flavanone producer strain capable of malonate assimilation (E2M), pACYC-MatB-MatC was introduced into E2. Flavone producer strain, E2V, was created by introducing pRSF-4CL-FSI plasmid[26] into E2.

The genes involved in the UDP-glucose biosynthesis from orotic acid of *E. coli* were PCR amplified according to the DNA sequences available in Genbank (Accession number NC000913.2, region 3813150–3813791 for *pyrE*, region 1339945–1340682 for *pyrF*, region 960424–961107 for *cmk*, and region 2642455–2642886 for *ndk*). The genes *pyrE*, *pyrF*, *cmk*, and *ndk*, encode the enzymes glucose-1-phosphate uridylyltransferase, phosphoglucomutase, orotate phosphoribosyltransferase, orotidine-5′-phosphate decarboxylase, cytidine monophosphate (CMP) kinase, and nucleoside diphosphate kinase, respectively. The PCR fragment of *pyrE* was inserted into vector pACYCDuet-1 between restriction sites *Bgl*II and *Xho*I resulting in plasmid pACYC-pyrE. Next, *pyrF* was cloned between *Sac*I and *Hin*dIII generating plasmid pACYC-pyrE-pyrF. Similarly, plasmids pCOLA-ndk and pCOLA-ndk-cmk were constructed by inserting *ndk* between *Bam*HI and *Hin*dIII and *cmk* between *Bgl*II and *Xho*I. *galU* (glucose-1-phosphate uridylyltransferase), with or without *pgm (*phosphoglucomutase), was inserted between *Eco*RV and *Kpn*I, and *Eco*RI and *Sal*I of pETDuet-1, respectively, to create pET-galU and pET-galU-pgm. Successful gene insertion was verified by restriction mapping, and the absence of undesired mutations introduced during PCR was verified by direct nucleotide sequencing.

***E. coli* BL21Star Mutant Generation through Gene Knockout.** Disruption of *udg* gene encoding for UDP-glucose dehydrogenase was carried out using the λ Red Recombinase/FLP system as previously described,[27] with the following modifications. Electrocompetent cells were prepared by washing the cell pellet twice with ice-cold sterile water then once with ice-cold 10% glycerol and finally

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concentrating in fresh, ice-cold 10% glycerol. The FRT-flanked kanamycin-resistant gene was PCR amplified with 40-nt extensions homologous to 5′ and 3′ regions of the *udg* coding sequence. Sterile LB was added immediately after pulsing in BioRad GenePulsar X-Cell system and then set to incubate at 37 °C without shaking for one hour before plating. To verify deletion of *udg*, mutants were streaked on LB plates to test for loss of all antibiotic resistance markers and further confirmed by PCR using isolated genomic DNA as template.

Recombinant Flavonoid Production by *E. coli.* For the production of recombinant flavanones, bacterial cultures were grown at 37 °C unless otherwise specified with orbital shaking at 300 rpm. For protein expression and heterologous flavanone production, overnight preinoculums were made in LB and then transferred into fresh LB medium at a starting A_{600} of 0.1 and grown until A_{600} reached 0.6. Expression of the recombinant genes was then performed by induction with 1 mM IPTG, and incubation continued at 30 °C for an additional 3 h. At the end of the incubation period, bacterial cells were collected by centrifugation and cultured in fresh medium (M9 or LB or TB) at A_{600} of 1.8–2.0. At that point, 3 mM phenylpropanoic acid substrates and 1 mM IPTG were added for inducing biotransformation. Incubation continued at 30 °C for 36 h prior to analysis of recombinant products. For E2M culture, 1 g/L sodium malonate was added twice within the period of 36 h. For E2V culture, 0.5 mM 2-oxoglutaric acid, 0.5 mM $FeSO_4$, and 0.5 mM sodium ascorbate were also added. FabB, FabF were repressed by the addition of specific inhibitor, the antibiotic cerulenin (Cayman Chemical) to the fermentative media after the induction period.[28] For the mutasynthesis of non-natural flavonoids, phenylpropanoic acid analogues were added at the concentration of 0.05 mM. The production of anthocyanins in *E. coli* was carried out as previously described.[23]

Flavonoid Analysis and Quantification. To analyze flavanone production, cell cultures were collected after 36 h. *E. coli* cells were separated through centrifugation, and the culture media were analyzed by high-performance liquid chromatography (HPLC), using an Agilent 1100 series instrument and a reverse-phase ZORBAX SB-C18 column (4.6 × 150 mm) maintained at 25 °C. Phenylpropanoic acids, flavanones and chrysin were separated by elution with an acetonitrile/water gradient at a flow rate of 1.0 mL/min under the following conditions: 10 to 40% acetonitrile (vol/vol) for 10 min, 40 to 60% acetonitrile (vol/vol) for 5 min, and 60 to 10% acetonitrile (vol/vol) for 2 min. The retention times under these conditions are caffeic acid, 6 min; *p*-coumaric acid, 7.5 min; eriodictyol 11 min; cinnamic acid, 12.1 min; naringenin, 12.8 min; crysin, 16.0 min; and pinocembrin, 16.3 min. HPLC conditions for apigenin and luteolin chromatography was 20 to 27% acetonitrile (vol/vol) for 45 min, 27 to 95% acetonitrile (vol/vol) for 30 s. Under these conditions, the retention times for luteolin and apigenin were 20.8 min and 33.2 min, respectively. HPLC conditions to

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Table 3. Production of Unnatural Flavonoids from E2 and E2V[a]

	productivity (mg/L)	
	−	+
Malonate Assimilation		
natural flavanone		
naringenin	42 ± 1	155 ± 12
eriodictoyl	11 ± 1	50 ± 7
pinnocembrin	29 ± 2	480 ± 23
Cerulenin		
unnatural flavanone		
5,7-dihydroxy-2′-fluoroflavanone	1.6 ± 0	3.6 ± 1
5,7-dihydroxy-4′-fluoroflavanone	2.5 ± 0	3.0 ± 1
5,7,2′-trihydroxyflavanone	3.4 ± 1	23.2 ± 1
5,7,3′-trihydroxyflavanone	2.6 ± 0	23.2 ± 1
4′-amino-5,7-dihydroxyflavanone	3.7 ± 1	12.4 ± 2
natural flavone		
crysin	0.3 ± 0	5 ± 1
apigenin	11 ± 2	110 ± 13
luteolin	0.4 ± 0	4 ± 1
unnatural flavone		
5,7-dihydroxy-2′-fluoroflavone	ND	0.4 ± 0
5,7-dihydroxy-4′-fluoroflavone	ND	0.4 ± 0
5,7,2′-trihydroxyflavone	0.2 ± 0	1 ± 0
5,7,3′-trihydroxyflavone	ND	ND
4′-amino-5,7-dihydroxyflavone	0.6 ± 0	2 ± 1

[a] The various recombinant *E. coli* strains expressing the flavanone and flavone biosynthetic pathways were employed for fermentation. Fermentation was carried out in modified M9 minimal medium with highest yields achieved after 40 h. ND indicates the compound was not found after extraction.

Table 4. Optimization of Cyanidin 3-*O*-Glucoside Production by Increasing the Intracellular Pool of UDP-Glucose[a]

E. coli strain	overexpressed gene(s)	deficient gene(s)	yield (mg/L)
JA	N/A	N/A	4 ± 1
YA	N/A	*galE, galT*	46 ± 3
YAP	*pgm, galU*	*galE, galT*	71 ± 8
YAN	*pgm, galU, ndk*	*galE, galT*	87 ± 9
YAC	*pgm, galU, ndk, cmk*	*galE, galT*	89 ± 9
YAY	*pgm, galU, ndk, cmk, pyrE, pyrF*	*galE, galT*	87 ± 8
AC	*cmk*	*galE, galT*	44 ± 7
AP	*pyrE, pyrF*	*galE, galT*	44 ± 6
AY	*cmk, pyrE, pyrF*	*galE, galT*	42 ± 6
YU	N/A	*galE, galT*, Δ*udg*	73 ± 8
YUP	*pgm, galU*	*galE, galT*, Δ*udg*	90 ± 9
YUN	*pgm, galU, ndk*	*galE, galT*, Δ*udg*	104 ± 7
YUC	*pgm, galU, ndk, cmk*	*galE, galT*, Δ*udg*	100 ± 3
YUY	*pgm, galU, ndk, cmk, pyrE, pyrF*	*galE, galT*, Δ*udg*	97 ± 6

[a] Various recombinant *E. coli* strains expressing translational protein 3A were employed for fermentation. Fermentation was carried out in modified M9 minimal medium (pH = 5.0) with no UDP-glucose in the culture medium where the highest yields were achieved after 17 h.

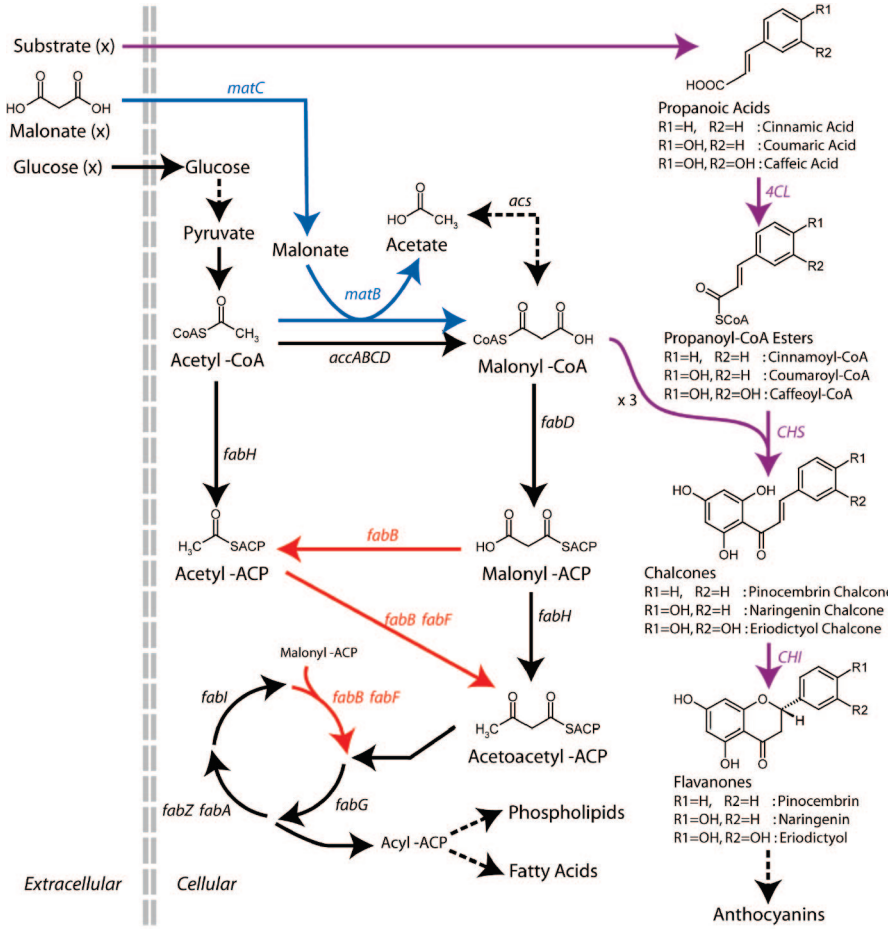

Figure 1. Engineered pathways for increased flavanone biosynthesis in recombinant *E. coli*. Both the recombinant pathways belonging to *R. trifolii*, including matB and matC (blue), and the recombinant plant biosynthetic pathway (purple), including 4-coumaroyl-CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI), are shown. Also indicated are the cerulenin specific targets FabB and FabF (red). Extracellular metabolites are denoted with (x).

separate non-natural flavanones were described previously.[4] Novel flavones were separated by elution with an acetonitrile/water gradient at a flow rate of 2.5 mL/min under the condition of 35 to 65% acetonitrile (vol/vol) for 20 min. The retention time for 4′-amino-5, 7-dihydroxyflavone, 5,7,2′-trihydroxyflavone, 5,7-dihydroxy-4′-fluoroflavone, and 5,7-dihydroxy-2′-fluoroflavone was 2.6 min, 3.6 min, 10.2 min, and 10.4 min, respectively. All flavanones and flavones were detected by monitoring absorbance at 290 and 340 nm respectively. Flavonoids derived from *E. coli* were identified by cochromatography and matching the retention time of HPLC profile and UV-absorbance profile with that of authentic compounds as previously described.[18] Flavonoid productions from the recombinant strains were presented as the average of three independent experiments. Anthocyanins were characterized as previously described.[25]

Results

Grafting of the Malonate Assimilation Operon in a Flavanone Producer *E. coli* Strain. Malonate utilization for flavonoid production improvement was accomplished by introducing *R. trifolii* MatB and MatC into the recombinant *E. coli* strain E2, which expressed plant 4CL, CHS, and CHI, to create strain E2M. The engineered malonate assimilation strain E2M was assessed for flavonoid production by culturing the recombinant strain in M9 minimal medium (with 1% glucose), and LB and TB rich media supplemented with 1 mM IPTG, 2 g/L sodium malonate, and 2 mM *p*-coumaric acid. Analysis of the biotransformation product after 36 h showed (Table 3 top) that the production of the flavanone naringenin in supplemented M9 minimal media reached up to 155 mg/L from E2M strain, which was 269% higher than the production from the parental strain, E2 (42 mg/L[24]). On the other hand, naringenin synthesis did not significantly improve when E2M was grown in LB or TB, which suggested the reduced functionality of the malonate assimilation ensembles in rich media (data not shown). The productivity of E2M to synthesize the two other natural flavanones, pinocembrin and eriodictyol from cinnamic acid, and caffeic acid, respectively was also evaluated (Table 3 top). From E2M culture, pinocembrin and eriodictyol production increased 1555% and 355% over the parental strain E2, reaching 480 mg/L and 50 mg/L, respectively. We also found that the activity of MatC was required for

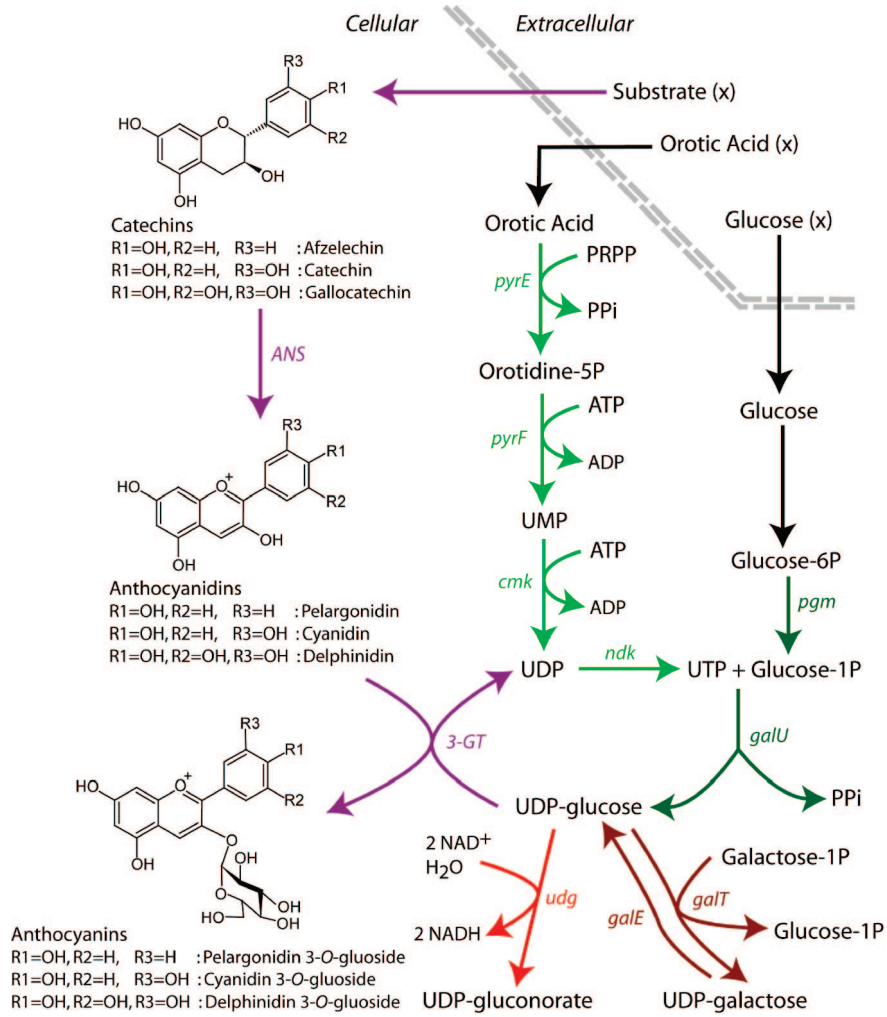

Figure 2. Engineered pathways for increase anthocyanin biosynthesis in recombinant *E. coli*. The recombinant plant biosynthetic pathway (purple) includes anthocyanin synthase (ANS) and 3-O-glucosyltransferase (3-GT). Overexpressed genes for orotic acid assimilation (green) and glucose-1-phosphate enhancement (dark green), *udg* gene deletion (red), and inactive genes *galE* and *galT* (dark red) are indicated with respective colors. Extracellular metabolites are denoted with (x).

malonate assimilation, since production levels did not increase with the overexpression of MatB alone (data not shown).

Inhibition of Fatty Acid Synthases Increased Flavonoid Production. In order to investigate the effect of Fab repression on flavonoid biosynthesis, the production of flavanones from strain E2 were evaluated by repressing FabB and FabF activities using various levels of cerulenin dosing. The maximum production of pinocembrin, naringenin, and eriodictyol was 710 mg/L, 186 mg/L, and 54 mg/L, respectively, when FabB/F were repressed (Figure 3A). Similarly, by partial repression of fatty acid pathway by cerulenin dosing, natural flavone synthesis increased significantly in the E2V strain over the control experiments. The production levels of chrysin, apigenin, and luteolin reached 5 mg/L, 110 mg/L, and 4 mg/L, respectively, which represented improvement of 1567%, 1000%, and 900% over the control strain (Table 3 bottom). For all strains, increased concentration levels of antibiotic (>1 mM), while further reducing growth rate, did not result in further flavonoid production increases (Figure 3). Supplementation with cerulenin decreased biomass accumulation in a dose-dependent manner and at the same time promoted the synthesis of the flavanones pinocembrin, naringenin, and eriodictyol with optimum production level seen when ~33% biomass was reduced (Figure 3B). In contrast, repression of FabB and FabF activity in E2M strain resulted in decreased flavonoid production upon malonate supplementation (data not shown). We also exploited the partial repression of fatty acid synthases to increase the capacity of the recombinant strains to mutasynthesize unnatural phenylpropanoic acid substrates. When fatty acid synthases were repressed, elevation in the synthesis of non-natural flavanones and flavones could be achieved (Table 3 bottom).

Orotic Acid Assimilation for Efficient Anthocyanin Biosynthesis. In the absence of experimental evidence that identifies the rate limiting step in the UTP biosynthetic pathway, the four enzymes responsible for UTP biosynthesis from orotic acid were sequentially overexpressed to improve UTP availability using the plasmid constructs pCOLA-ndk, pCOLA-ndk-cmk, and pACYC-pyrE-pyrF. The generated

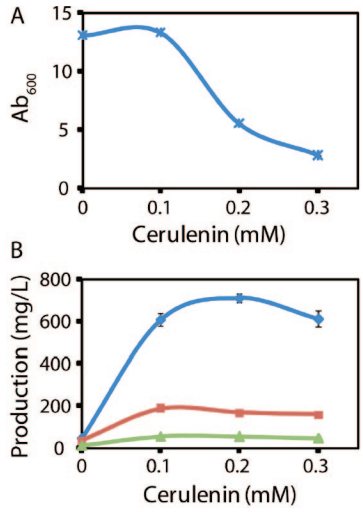

Figure 3. Final production dose–response curves for both growth (A) and production (B) of naringenin (red), eriodictyol (green), and pinocembrin (blue) in strain E2 due to treatment with cerulenin. All trials shown use the supplemented M9 minimal media.

plasmid constructs were transformed into the previously constructed strain YA,[25] and the resulting recombinant strain was tested for cyanidin 3-*O*-glucoside production under 0.1 mM orotic acid supplementation. The expression of *ndk* and orotic acid supplementation resulted in anthocyanin production of 87 mg/L. Overexpression of *cmk*, *pyrE* and *pyrF* did not lead to further improvements in productivity, nor did the inclusion of *cmk*, *pyrE* and *pyrF* overexpressions with the *ndk* strain (Table 4).

Knockout Mutant for Further Anthocyanin Production Improvement. Our primary host strain BL21Star is already engineered to have inactive gene products from both *galE* and *galT* unlike the *E. coli* JM109(DE3) host cell line, therefore an additional *udg* BL21Star knockout mutant was developed for comparison. Anthocyanin production was assayed in both the resulting mutant strains, YU, and the control strain, YA, by shake flask fermentations both harboring the ANS (anthocyanidin synthase) and 3-GT (3-*O*-glucosyltransferase) fusion protein. As shown in Table 4, strain YU yielded an increased production up to 73 mg/L cyanidin 3-*O*-glucoside, which was 61% higher when compared to the control. This improved phenotype was then cotransformed with the cloned overexpression vectors described above. The resulting strains produced cyanidin 3-*O*-glucoside up to 104 mg/L (Table 4). Similarly, around 113 mg/L of pelargonidin 3-*O*-glucoside was produced when afzelechin was used as the precursor flavan-3-ol. As before, the additional overexpression of *cmk*, *pyrE* and *pyrF* did not lead to further yield improvement (Table 4). Intracellular accumulation of anthocyanin 3-*O*-glucosides contributed to more than 50% of the total yield (data not shown).

Discussion

E. coli has been utilized as drug factory for the production of a diverse array of important pharmaceuticals yet, in most cases, the productivity is greatly limited by the low availability of intracellular precursors. In this study, we identified the limitations on precursor availability for the high-level flavanone and anthocyanin production from engineered *E. coli* strains expressing the plant flavonoid biosynthetic pathways. Thereafter, we developed several metabolic and genetic engineering strategies to allow high-yield flavonoid production by improving the intracellular pool of malonyl-CoA and UDP-glucose.

The introduction of a malonate assimilation pathway from *R. trifolii* allowed for the assimilation of an exogenous carbon source to directly synthesize malonyl-CoA. This strategy bypassed the natural metabolism of malonyl-CoA from glucose, which involves several biochemical steps (Figure 1). The engineered bicistronic expression of MatB and MatC through individual regulation by the T7*lac* promoter, as suggested from prior studies[29] and in contrast to the natural monocistronic construct found in *R. trifoli*, allowed optimized protein expression. The introduction of this malonate assimilation pathway increased natural flavanone synthesis up to 250%. The threshold amount of malonyl-CoA in *E. coli* is attributed to the complexity of fatty acid metabolism. In this case, fatty acid synthases mediated the conversion of malonyl-CoA into acetyl-CoA. To circumvent the depletion of malonyl-CoA, we explored the effect of inhibiting fatty acid synthases toward flavonoid synthesis. The inhibition of fatty acid biosynthesis by cerulenin increased the production levels of flavonoids to over 900%. This result confirmed that the strong metabolic channeling of carbon toward fatty acids is the major competitive step in recombinant flavonoid biosynthesis. Higher dosing of cerulenin, while reducing growth rate, did not result in flavonoid production increases. This finding suggests that extremely high malonyl-CoA is detrimental to flavonoid biosynthesis. In fact, it has also been reported that FabF overexpression, while increasing malonyl-CoA levels 30–40%, resulted in cell death 12 min after induction of the overexpression.[30] Therefore, by systematically repressing FabB and FabF with a specific inhibitor, we approached the limit of malonyl-CoA saturation in the cell. In addition we have shown for the first time that by increasing the flux leading to an adjust precursor (malonyl-CoA), the increased bioconversion of non-natural substrates (phenylpropanoic acid analogues) through an engineered plant biosynthetic pathway resulted in high-level non-natural flavonoids from *E. coli*.

In previous work, it was shown that UDP-glucose availability is a limiting step in the biosynthesis of another class of flavonoids, namely, anthocyanins.[25] For that reason, several metabolic engineering strategies were developed to

(29) Hwang, E. I.; Kaneko, M.; Ohnishi, Y.; Horinouchi, S. Production of plant-specific flavanones by *Escherichia coli* containing an artificial gene cluster. *Appl. Environ. Microbiol.* **2003**, *69* (5), 2699–2706.

(30) Subrahmanyam, S.; Cronan, J. E., Jr. Overproduction of a functional fatty acid biosynthetic enzyme blocks fatty acid synthesis in Escherichia coli. *J. Bacteriol.* **1998**, *180* (17), 4596–602.

improve the UDP-glucose intracellular concentration. The approaches included augmenting the metabolic flux to UDP-glucose synthesis by engineering both pentose phosphate pathway and nucleotide synthesis pathway through gene-overexpression and elimination of the endogenous UDP-glucose consumption pathway. The highest anthocyanin production of up to 113 mg/L was achieved by the overexpression of *ndk* and deletion of *udg*. Further overexpressions of *cmk*, *pyrE* and *pyrF* did not improve production levels, which suggested that *ndk* is the limiting step in UTP synthesis.

This study demonstrated the extent of engineering strategies that were required to overproduce flavonoids both in the upper (flavanones) and in the lower (anthocyanins) pathways. Clearly, the integration of upper and lower pathways will rely on high cofactor and precursor availability within the recombinant host in order to achieve the optimum anthocyanin synthesis starting from phenylpropanoic acids. In general, the development of overproducer strains offers a viable approach for large-scale production of core flavonoid molecules while the creation of unnatural flavonoids through the recombinant engineering strategy could also shed light on drug design and development.

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