Exploring Recombinant Flavonoid Biosynthesis in Metabolically Engineered *Escherichia coli*

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Flavonoids are important plant-specific secondary metabolites synthesized from 4-coumaroyl coenzyme A (CoA), derived from the general phenylpropanoid pathway, and three malonyl-CoAs. The synthesis involves a plant type III polyketide synthase, chalcone synthase. We report the cloning and coexpression in Escherichia coli *of phenylalanine ammonia lyase, cinnamate-4-hydroxylase, 4-coumarate:CoA ligase, and chalcone synthase from the model plant* Arabidopsis thaliana*. Simultaneous expression of all four genes resulted in a blockage after the first enzymatic step caused by the presence of nonfunctional cinnamate-4-hydroxylase. To overcome this problem we fed exogenous 4-coumaric acid to induced cultures. We observed high-level production of the flavanone naringenin as a result. We were also able to produce phloretin by feeding cultures with 3-(4-hydroxyphenyl)propionic acid. Feeding with ferulic or caffeic acid did not yield the corresponding flavanones. We have also cloned and partially characterized a new tyrosine ammonia lyase from* Rhodobacter sphaeroides*. Tyrosine ammonia lyase was substituted for phenylalanine ammonia lyase and cinnamate-4-hydroxylase in our* E. coli *clones and three different growth media were tested. After 48 h induction, high-level production (20.8 mg L^{-1}) of naringenin in metabolically engineered* E. coli *was observed for the first time.*

Introduction

Flavonoids are ubiquitous plant natural products that play a variety of roles in plants, such as UV protection,[1] defense against pathogens,[2] and coloration.[3] The uncovering of an increasing number of health benefits associated with flavonoids present in fruits, vegetables, red wine, and green tea has resulted in an explosion of research on the medicinal properties of these compounds over the last few years.[4] The proven medicinal activities of flavonoid compounds range from scavenging of harmful oxygen species, enzyme inhibition, and anti-inflammatory and estrogenic activities, to cytotoxic antitumor activities.[5]

The recognition of flavonoids as health-promoting nutraceuticals has also spurred on research attempting to elucidating the complex metabolic networks of flavonoid biosynthesis with the aim of enhancing and altering flavonoid composition in dietary plants.[6] Most plant flavonoid biosynthetic enzymes that have been characterized on a molecular level were previously individually expressed in *Escherichia coli* as functional enzymes.[7–9] Coexpression or engineering of these plant flavonoid biosynthetic genes in bacteria has not been reported.

Flavonoids are synthesized from an activated phenylpropanoid starter unit and three malonyl coenzyme A (malonoyl-CoA) extender units. Phenylpropanoids are phenolic acids, such as 4-coumaric, caffeic, and ferulic acid (Scheme 1), that are used in the formation of lignins, coumarins, and other plant natural products, including flavonoids.[10] The first step in phenylpropanoid biosynthesis is deamination of L-phenylalanine by phenylalanine ammonia lyase to produce *trans*-cinnamic acid. *trans*-Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase at the *para* position of the benzyl ring to form 4-coumaric acid, which is then activated by 4-coumaroyl:CoA ligase to make 4-coumaroyl-CoA. Naringenin chalcone is synthesized from a single activated 4-coumaroyl-CoA starter unit by sequential addition of three acetate extender units derived from malonyl-CoA. These addition steps are catalyzed by a type III polyketide synthase, chalcone synthase.[11] Naringenin chalcone is then converted into the three-ringed flavanone structure naringenin. This conversion occurs spontaneously in vitro or is catalyzed by the enzyme chalcone isomerase in vivo.[12]

While we were preparing this manuscript another group published[13] a report on the construction of a hybrid flavonoid biosynthetic pathway in *E. coli* from a combination of yeast, bacterial, and plant genes. Our approach differs from that described in this previous report because we engineered a flavonoid biosynthetic pathway in *E. coli* by using only *Arabidopsis thaliana* genes. We also report the cloning and partial in vivo characterization of a new tyrosine ammonia lyase from *Rhodobacter sphaeroides*. Some plant PAL isoenzymes are known to deaminate both phenylalanine (PAL activity) and tyrosine (TAL activity).[14] The first example of a bacterial TAL was cloned recently from *Rba. capsulatus*. This enzyme is the first PAL/TAL-type ammonia lyase to be found in bacteria.[15] We substituted TAL from *Rba. sphaeroides* 158 for *Arabidopsis* PAL and C4H and were able to detect for the first time high-level production of naringenin in *E. coli*. We measured a 250-fold increase in production over that previously observed when no tyrosine was fed to the culture medium. This result opens up the possibility of further metabolic engineering of flavonoid biosynthetic pathways in recombinant *E. coli*.

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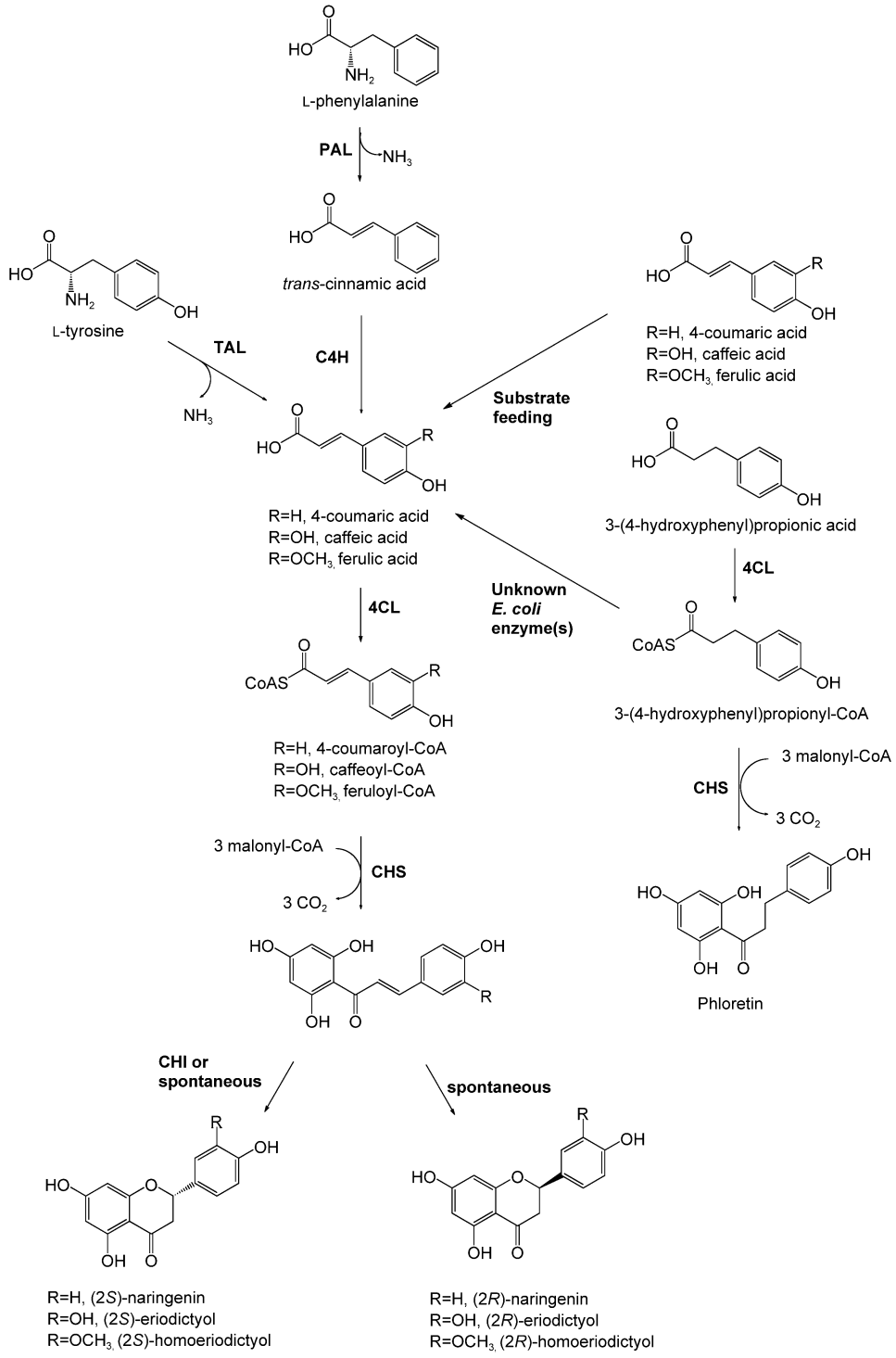

Scheme 1. *Engineered flavonoid biosynthetic pathway in* E. coli. *Substrates can be biosynthetically derived or fed directly into recombinant* E. coli *expressing 4CL and CHS. Enzyme abbreviations: PAL, phenylalanine ammonia lyase (*Arabidopsis thaliana*); C4H, cinnamate-4-hydroxylase (*A. thaliana*); 4CL, 4-coumaroyl:CoA ligase (*A. thaliana*); CHS, chalcone synthase (*A. thaliana*); TAL, tyrosine ammonia lyase (*Rhodobacter sphaeroides*); CHI, chalcone isomerase.*

Results

Cloning and assembly of the *Arabidopsis* naringenin pathway in *E. coli*

PAL and C4H were cloned into the medium-copy-number plasmid pACMod (Table 1) under the control of the arabinose promoter (pACMod-PAL/C4H). 4CL and CHS were cloned into the high-copy number plasmid pBADMod2, again with the arabinose promoter (pBADMod2-4CL/CHS). This modified pBAD plasmid also contains the arabinose repressor, AraC, which is important for controlling gene expression from the arabinose promoter.[19] These two plasmids (pACMod-PAL/C4H and pBADMod2-4CL/CHS) were cotransformed into *E. coli* BW27784, a strain that overexpresses a chromosomal low-affinity, high-capacity arabinose permease (AraE).[17] After 24 h induction, culture supernatants and pellets of cultures grown in modified M9, Luria-Bertani (LB), or Terrific broth (TB) medium were extracted and analyzed by HPLC. The only product detected in the culture supernatants and cell pellets was *trans*-cinnamic acid (Figure 1B), with the majority found in the supernatants. This result indicates the presence of a blockage after the first enzymatic step catalyzed by PAL (Scheme 1). When we checked the protein expression levels by SDS-PAGE, we found recombinant proteins (PAL, C4H, 4CL, and CHS) in both the soluble and insoluble fractions (data not shown).

Since *trans*-cinnamic acid was not hydroxylated to 4-coumaric acid by the second enzyme in the pathway, C4H, it appeared that this cytochrome P450 monooxygenase is nonfunctional in *E. coli*. To investigate whether the blockage was in fact caused by nonfunctional C4H and whether the subsequent enzymes in the pathway were functional, exogenous 4-coumaric acid was fed at induction to recombinant *E. coli* expressing pACMod-PAL/C4H + pBADMod2-4CL/CHS grown in modified M9 medium. After 24 h induction, the culture was harvested and naringenin was detected by HPLC (Figure 1C) in both the culture supernatant and cell pellet, with the majority in the culture supernatant. The naringenin was identified by LC-MS/

Table 1. *Strains and plasmids used in this study.*

Strain or plasmid	Relevant properties or genotype	Source
Strains		
E. coli JM109	*recA1 supE44 endA1 hsdR17* ($r_K^- m_K^+$) *gyrA96 relA1 thi* Δ(*lac-proA*B)[F′*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	[16]
E. coli BW27784	*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR514* Δ*(araBAD)567* Δ*(rhaBAD)568* Δ *(araFGH)* ϕ (Δ *araEp* P_{CP18}-*ara*E)	[17]
Rba. capsulatus 1710	type strain	DSMZ[a]
Rba. sphaeroides 158	type strain	DSMZ[a]
Plasmids		
pUCMod	cloning vector, constitutive *lac* promoter, Amp^r	[18]
pACMod	cloning vector, Tet^r, Cm^r	[18]
pBADMod1	cloning vector from pBAD-Thio/TOPO, Amp^r	this study
pBADMod2	cloning vector, Amp^r	this study
pBADMod1-PAL	Arabinose inducible PAL from *A. thaliana*	this study
pBADMod1-C4H	Arabinose inducible C4H from *A. thaliana*	this study
pBADMod1–4CL	Arabinose inducible 4CL from *A. thaliana*	this study
pBADMod1-CHS	Arabinose inducible CHS from *A. thaliana*	this study
pACMod-PAL/C4H	Arabinose inducible PAL and C4H, Tet^r	this study
pBADMod2-4CL/CHS	Arabinose inducible 4CL and CHS, Amp^r	this study
pUCMod-TAL	constitutively expressed TAL from *Rba. sphaeroides*	this study
pACMod-TAL	constitutively expressed TAL from *Rba. sphaeroides*, Cm^r	this study

[a] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

MS (*m*/*z*: 271.1) through comparison of the obtained fragmentation pattern with that of an authentic standard and literature data.[20] No residual 4-coumaric acid was detected, which indicates that 4-coumaric acid can be efficiently transported and metabolized by *E. coli* expressing functional 4CL and CHS. High levels of *trans*-cinnamic acid were detected because functional PAL was still present in the assembled four-gene pathway.

To confirm that 4CL and CHS function in a background devoid of PAL and C4H, 4-coumaric acid was fed in the same way to *E. coli* expressing only the pBADMod2-4CL/CHS plasmid. Naringenin was produced and no *trans*-cinnamic acid could be detected (Figure 1D) by HPLC or LC-MS after 24 h induction. No naringenin was detected in control cultures containing pBADMod2-4CL/CHS not fed with 4-coumaric acid (data not shown).

Feeding with additional phenylpropanoid precursors

As with 4-coumaric acid, feeding experiments were performed with caffeic, ferulic, and 3-(4-hydroxyphenyl)propionic acids. These compounds were fed to cultures of *E. coli* cells harboring pBADMod2-4CL/CHS to examine the substrate specificities of 4CL and CHS in vivo.

Caffeic and ferulic acids were not converted into the corresponding chalcone or flavanone (eriodictyol and homoeriodictyol, respectively) in modified M9 or TB medium, as determined by HPLC (data not shown). However, HPLC and LC-MS analysis showed that cultures fed with 3-(4-hydroxyphenyl)propionic acid produced both the expected product, phloretin (*m*/*z*: 273.1), and the 4-coumaric acid product (naringenin; Scheme 1) in equal but small amounts after 24 h cultivation. A large amount of 4-coumaric acid (*m*/*z*: 163.0) had accumulated after this time period but no 3-(4-hydroxyphenyl)propionic acid was detected (Figure 2).

To determine whether phloretin was converted into naringenin by *E. coli* or during the extraction process, phloretin was fed at induction to control cultures containing an empty vector (pBADMod2). After 24 h, the culture was extracted and found to contain phloretin but no detectable naringenin. The extraction was carried out with the pH value adjusted to 9.0 and without any such adjustment; both extractions gave identical results. Next, we tested whether *E. coli* metabolizes 3-(4-hydroxyphenyl)propionic acid into 4-coumaric acid by feeding 3-(4-hydroxyphenyl)propionic acid to control *E. coli* cultures containing the empty vector pBADMod2. After 24 h, no 4-coumaric acid was detected, only 3-(4-hydroxyphenyl)propionic acid was found. *E. coli* cultures expressing either 4CL or CHS alone were individually fed with 3-(4-hydroxyphenyl)propionic acid. The *E. coli* cells expressing only 4CL converted 3-(4-hydroxyphenyl)propionic acid into 4-coumaric acid, which suggests there may be an unknown *E. coli* enzyme that acts on the CoA ester of 3-(4-hydroxyphenyl)propionic acid. In the presence of CHS alone, only 3-(4-hydroxyphenyl)-propionic acid was detected after induction; no conversion was observed.

Cloning and expression of *Rba. sphaeroides* TAL

We attempted to clone the recently described TAL from *Rba. capsulatus*[15] to overcome the blockage caused by the lack of function of C4H in *E. coli*. The *Rba. capsulatas* TAL is known to convert tyrosine into 4-coumaric acid, which is required for the formation of the chromophore of a photoactive yellow protein.[21] We repeatedly failed to amplify a product of the expected size from genomic DNA by following the procedures described in the literature. We then conducted a BLAST search with the available *Rba. capsulatus* TAL sequence as a query and identified a hypothetical protein (GenBank accession no. ZP 00005404) from *Rba. sphaeroides* with 51% amino acid identity with the published TAL. We amplified the gene corresponding to this protein from genomic DNA and cloned it into pUCMod for expression under control of a constitutive *lac* promoter. *E. coli* cells containing pUCMod-TAL were able to produce 4-coumaric acid but not *trans*-cinnamic acid (the deamination products of tyrosine and phenylalanine, respectively), as determined by HPLC and LC-MS. Production of 4-coumaric

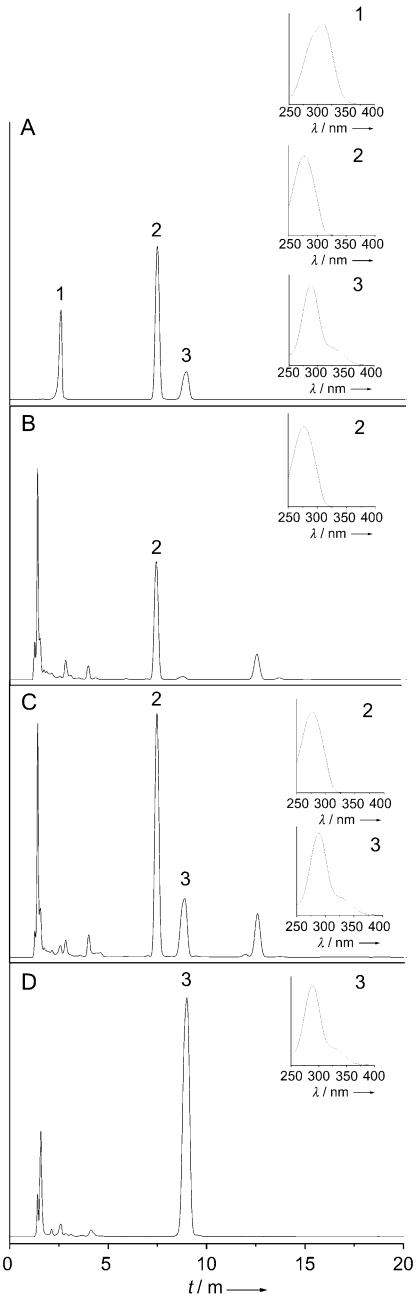

Figure 1. *HPLC analysis of extracts from supernatants of cultures containing* E. coli *cells coexpressing* Arabidopsis *PAL, C4H, 4CL, and CHS. The cells were cultured in modified M9 medium and analyzed after 24 h induction. A) Standard compounds, 4-coumaric acid (**1**),* trans*-cinnamic acid (**2**), and naringenin (**3**). B)* E. coli *pAC-PAL/C4H + pBAD-4CL/CHS. C)* E. coli *pAC-PAL/C4H + pBAD-4CL/CHS fed with 4-coumaric acid. D)* E. coli *pBAD-4CL/CHS fed with 4-coumaric acid. Absorbance was monitored at 290 nm. The insets show the UV/Vis spectra of the compounds with the indicated HPLC peaks. The absorbance maxima of 4-coumaric acid,* trans*-cinnamic acid, and naringenin are at 310, 275, and 290 nm, respectively.*

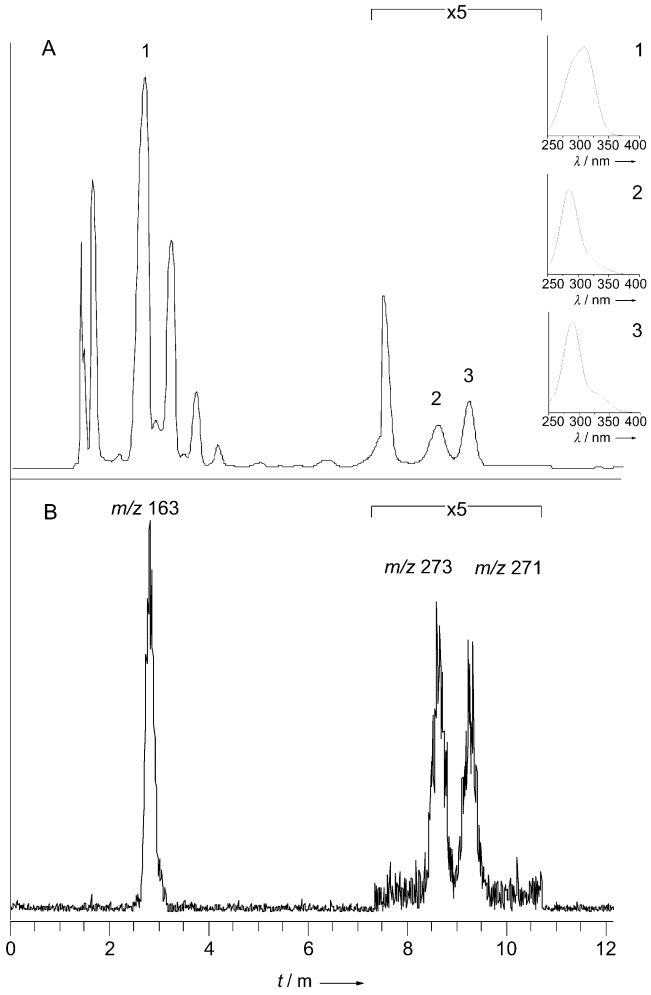

Figure 2. *Phloretin production in* E. coli *cells fed with 3-(4-hydroxyphenyl)propionic acid. A) HPLC chromatogram showing the accumulation of 4-coumaric acid (**1**) and production of both phloretin (**2**) and naringenin (**3**). B) Selective ion chromatogram of the culture fed with 3-(4-hydroxyphenyl)propionic acid with peaks at the masses of 4-coumaric acid, phloretin, and naringenin. Absorbance was monitored at 290 nm. The insets show the UV/Vis spectra of the compounds with the indicated HPLC peaks. The absorbance maximum of phloretin is at 287 nm.*

acid was highest in TB medium, followed by modified M9 then LB medium (data not shown).

We subcloned TAL into pACMod to allow coexpression with pBADMod2-4CL/CHS in *E. coli*. Transformation of pACMod-TAL into *E. coli* resulted in the production of 4-coumaric acid (2.30 mg L^{-1}), found in the culture supernatant after 24 h cultivation in modified M9 medium (Figure 3 B).

Production of naringenin in *E. coli* through a three-gene hybrid pathway

To establish a functional hybrid pathway for naringenin production, pACMod-TAL and pBADMod2-4CL/CHS were cotransformed into *E. coli* BW27784. *E. coli* cells expressing this three-gene pathway (TAL + 4CL + CHS) were grown in modified M9, LB, or TB medium and the culture medium was extracted after 24 h induction (Figure 3 C and data not shown). Naringenin was detected in all culture supernatants and cell pellets examined, with the majority found in the culture supernatants.

E. coli cells expressing the TAL-4CL-CHS hybrid pathway were cultured in modified M9 or TB medium to monitor narin-

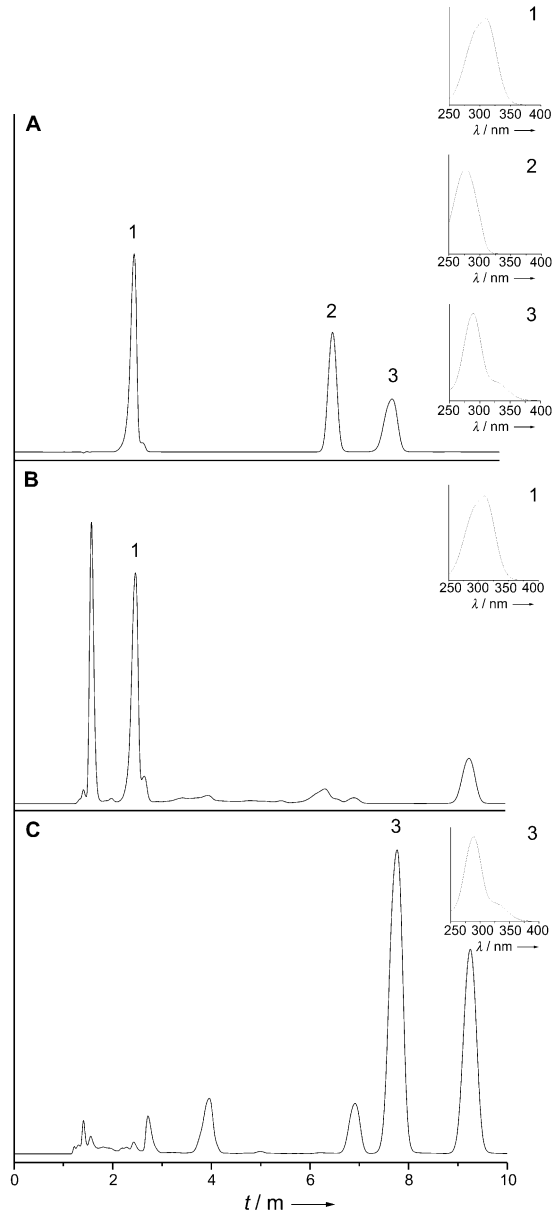

Figure 3. *HPLC analysis of extracts from supernatants of cultures containing* E. coli *transformants expressing* Rba. sphaeroides *TAL alone or together with* Arabidopsis *4CL and CHS in modified M9 medium after 24 h induction. A) Standard compounds 4-coumaric acid (**1**),* trans*-cinnamic acid (**2**), and naringenin (**3**). B)* E. coli *pAC-TAL + pBADMod2. C)* E. coli *pAC-TAL + pBAD-4CL/CHS. Absorbance was monitored at 290 nm. The insets show the UV/Vis spectra of the compounds with the indicated HPLC peaks.*

genin production levels during growth. Samples were removed from the cultures 12 h after induction with arabinose for quantification of naringenin by HPLC. Naringenin production was highest in TB and the product was found almost exclusively in the culture media, which accounted for more than 90% of the total amount produced. Naringenin was not detected in either medium at induction but the amount present increased over time. In TB medium (Figure 4A), 1.45, 7.65, 13.5, and 20.8 mg L^{-1} naringenin was detected 12, 24, 36, and 48 h after induction, respectively. In modified M9 medium (Figure 4B), 0.93, 4.89, 7.39, and 7.53 mg L^{-1} naringenin was detected at the same time intervals after induction. The amount of product in the cell pellets reached a maximum 36 h after induction in modified M9 medium (0.43 mg L^{-1}) and 48 h after induction in TB (0.73 mg L^{-1}) and accounted for 5.8 and 2.9% of the total yield at those times, respectively.

Discussion

Previous work has demonstrated that the enzymes involved in flavonoid biosynthesis in *Arabidopsis* interact both in vitro and in vivo.[22] In an effort to conserve these interactions in vivo in our heterologous system, we coexpressed all *Arabidopsis* enzymes necessary for naringenin production in *E. coli*. We determined that the entire pathway was functional, except the step catalyzed by cinnamate-4-hydroxylase. C4H is a P450 cytochrome monooxygenase (CYP73A5) and this class of enzyme requires a complementary reductase for activity (reduced nicotinamide-adenine dinucleotide phosphate (NADPH) reductase). Recombinant C4H activity has previously only been demonstrated in vitro with a complementary NADPH reductase enzyme.[7,23] The *E. coli* flavoproteins flavodoxin (Fld) and NADPH flavodoxin reductase (Fpr) have been shown to function as an electron-transfer system for some microsomal cytochrome P450 enzymes both in *E. coli* and in vitro,[24] but were not sufficient to activate C4H.

We were able to overcome the failure of this enzymatic step (hydroxylation of *trans*-cinnamic acid to 4-coumaric acid by C4H) by cloning a new bacterial tyrosine ammonia lyase from *Rba. sphaeroides* 158. This enzyme is only the second bacterial tyrosine-specific ammonia lyase to be isolated and characterized.[15] In contrast to known eukaryotic PAL isoenzymes with TAL activity, which include the yeast PAL used in a previous study on flavonoid production in *E. coli*, this new bacterial TAL appears to be specific for tyrosine; only 4-coumaric acid accumulates in recombinant *E. coli* cells, no *trans*-cinnamic acid is detectable (Figure 3B). Further characterization of this TAL in vitro is necessary to determine the kinetic constants of the enzyme and to test its substrate specificity. By substituting TAL for PAL + C4H to produce a shortened naringenin biosynthetic pathway consisting of TAL, 4CL, and CHS, we were able to produce significant amounts (20.8 mg L^{-1}) of the flavonoid naringenin in recombinant *E. coli*. The naringenin was found almost exclusively in the culture supernatant and the amount produced is 250 times higher than that reported[13] when the medium is not supplemented with tyrosine.

We were also able to produce naringenin by feeding exogenous 4-coumaric acid to the shortened naringenin biosynthetic pathway and we extended this approach to produce phloretin, a compound not usually formed through this pathway. The substrate specificity of *Arabidopsis* 4CL isoforms has been investigated in vitro with several substrates, such as caffeic and ferulic acid,[9] but not with 3-(4-hydroxyphenyl)propionic acid. We tested these same substrates by feeding them to our system in vivo and found that the products expected from caffeic and ferulic acid were not formed. This outcome is possibly

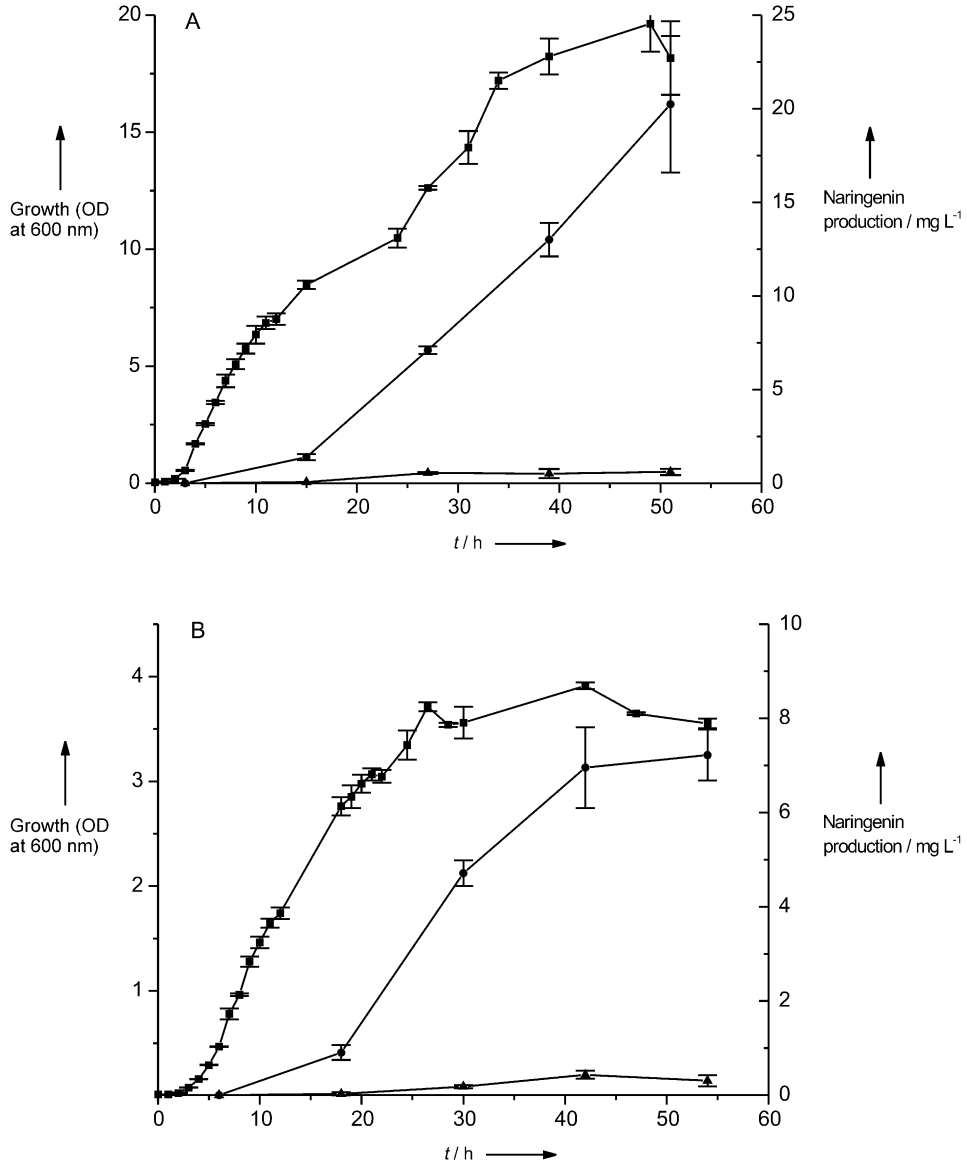

Figure 4. *Growth and naringenin production of recombinant* E. coli *expressing* Rba. sphaeroides *TAL together with* Arabidopsis *4CL and CHS in TB (A) and modified M9 (B) medium. Squares represent growth, circles and triangles represent naringenin detected in the culture supernatant and cell pellets, respectively. Data points are mean values calculated from measurements taken from three independent cultures.*

the result of the substrate specificity of the *Arabidopsis* CHS isoenzyme used, or limited transport of the substrates into cells. We were able to produce small but detectable amounts of phloretin from 3-(4-hydroxyphenyl)propionic acid by using this feeding strategy. The concurrent production of equally small amounts of naringenin and the build-up of 4-coumaric acid indicate, however, that this process is not efficient in vivo. We determined that the 4-coumaric acid produced in cultures fed with 3-(4-hydroxyphenyl)propionic acid results from the action of 4CL and unknown *E. coli* enzyme(s) that convert activated 3-(4-hydroxyphenyl)propionic acid into 4-coumaric acid. *E. coli* is known to degrade a variety of aromatic acids, although some pathways are not complete in K-12 laboratory strains.[25] Furthermore, a soluble thioesterase present in *E. coli* has been reported to catalyze rapid hydrolysis of aromatic coenzyme A esters (including 4-coumaroyl-CoA) to the corresponding free acids and thus prevent their accumulation.[26] We do not know why 4-coumaric acid accumulated in our cultures without a concurrent increase in naringenin production. A build-up of 4-coumaric acid was not seen in the same recombinant *E. coli* cultures when they were not fed 3-(4-hydroxyphenyl)propionic acid or were fed 4-coumaric acid. One possible explanation is that the activated 3-(4-hydroxyphenyl)propionic acid substrate or the phloretin product inhibits CHS activity in vivo. This hypothesis remains to be investigated.

We have demonstrated that high-level production of flavonoids in a bacterial host is possible. This result opens the door to further extension of the flavonoid pathway in *E. coli* with the many known biosynthetic genes to produce pathways for the synthesis of significant quantities of other important plant-derived flavonoid compounds, such as flavonols, flavones, isoflavonoids, condensed tannins, and anthocyanin pigments. We have also demonstrated that exogenously supplied phenylpropionic acids are readily taken up and converted into the corresponding flavonoids by recombinant *E. coli* cultures. Thus, phenylpropionic acids, which are abundantly available from agricultural waste products and are currently exploited in processes such as microbial vanillin production,[27] could serve as inexpensive precursors for more highly valued flavonoid compounds. In vivo feeding with exogenous precursor compounds will provide an elegant method to determine the catalytic functions of CoA ligases and type III polyketide synthases, isoenzymes and engineered variants of the known enzymes 4CL and CHS, and also the large number of as yet unidentified bacterial and plant homologues uncovered in genome sequencing projects.

Experimental Section

Chemicals: Caffeic acid, ferulic acid, and 3-(4-hydroxyphenyl)-propionic acid were purchased from Sigma Aldrich (St. Louis, MO). Naringenin, 4-coumaric acid, phloretin, and arabinose were pur-

chased from ICN (Aurora, OH). *trans*-Cinnamic acid was obtained from Acros Organics (Morris Plains, NJ). All solvents used were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA). HPLC-grade water was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). T4 DNA ligase and Vent DNA polymerase were obtained from New England Biolabs (Boston, MA). Restriction enzymes were purchased from NEB or Promega (Madison, WI). SuRE/Cut buffers from Roche (Indianapolis, IN) were used as the restriction enzyme buffers.

Strains and culture conditions: All cloning and DNA manipulation was carried out in *E. coli* JM109 cells by using standard techniques.[28] Cells were grown at 30 °C with shaking at 300 rpm. Following sequencing, plasmids were transformed into *E. coli* strain BW27784[17] (Table 1), provided by the *E. coli* Genetic Stock Center (New Haven, CT). *Rba. capsulatus* (DSM no. 1710) and *Rba. sphaeroides* (DSM no. 158) were obtained from the DSMZ (Braunschweig, Germany). *Rba. capsulatus* was grown anaerobically at 30 °C under direct light in modified Van Niel's medium (ATCC medium 1676) for more than 5 days. *Rba. sphaeroides* 158 was grown aerobically at 30 °C in LB medium for 3 days. Genomic DNA was prepared with a Wizard Genomic DNA kit (Promega, Madison, WI). *E. coli* cells harboring either the *Arabidopsis* pathway (pACMod-PAL/C4H + pBADMod2-4CL/CHS) or the TAL pathway (pACMod-TAL + pBADMod2-4CL/CHS) were grown in modified M9, LB, or TB medium, supplemented with tetracycline (12.5 mg mL^{-1}) or chloramphenicol (50 mg mL^{-1}) and carbenicillin or ampicillin (100 mg mL^{-1}), to an optical density of 0.4–0.6 at 600 nm. The cultures were induced with arabinose (0.2% *m/v*). M9 medium was modified by the addition of yeast extract (1.25 g L^{-1}) and glycerol (0.5% *v/v*) to standard M9 medium.[28]

Plasmid construction and gene cloning: pBADMod1 was constructed from pBAD/Thio-TOPO (Invitrogen, Carlsbad, CA) by elimination of the *Nco*I/*Pme*I fragment by long-range PCR. Primers with the sequences 5′-*GGCGCGCC*TTAAACAAAATTATTTCTAG-3′ and 5′-*TAATTAA*GGTCTCCAGCTTGGCTG-3′ were used to introduce unique *Asc*I and *Pac*I sites downstream of the arabinose promoter. pBADMod2 was constructed in the same way, with primers (5′-*GGTACCCTCGAGGTTTAAACAAGCTT*CGCTTCTCTGAGTAGGAC-3′ and 5′-*CCATGGGCGGCCGCGAATTCGTCGAC*CTCTGAATGGCGGGAG-3′) selected to eliminate the arabinose promoter and terminator and introduce a multiple cloning site. pUCMod and pACMod have been described previously.[18]

Phenylalanine ammonia lyase (Genbank accession no. AY303128), cinnamate-4-hydroxylase (U71080), 4-coumaroyl:CoA ligase (U18675), and chalcone synthase (AF112086) were cloned from a pFL61 *Arabidopsis thaliana* cDNA library purchased from the American Type Culture Collection (ATCC, Manassas, VA; no. 77500). The cDNA sequences included forward primers containing a 5′-*Asc*I site, followed by an optimized Shine–Dalgarno sequence (5′-*AGGAG*GATTACAAA**ATG**-3′) and the start codon for each gene, then an additional 10–15 nucleotides corresponding to the respective gene sequences. Reverse primers containing a *Pac*I site for directional cloning into pBADMod1 were used. PCR was carried out with Vent polymerase under the following conditions: 94 °C for 2 minutes, 30 cycles at 94 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 1 minute, and a final extension step at 72 °C for 4 minutes. PAL and C4H were subcloned, along with the arabinose promoter, from pBADMod1 into pACMod by using the *Nco*I and *Eco*RI sites, respectively. This process led to pACMod-PAL/C4H. 4CL and CHS were subcloned into the *Nco*I and *Xho*I sites of pBADMod2, respectively, to create pBADMod2-4CL/CHS.

Tyrosine ammonia lyase (hypothetical protein no. ZP 00005404) was cloned from *Rba. sphaeroides* 158 genomic DNA into the *Xba*I/*Sma*I sites of pUCMod by using the primers described above (forward primer with a Shine–Dalgarno sequence and start codon). The PCR conditions were the same as those given above except that dimethylsulfoxide (10% *v/v*) and betaine monohydrate (final concentration 1 M) were added. TAL was later subcloned into the *Bam*HI site of pACMod to create pACMod-TAL.

Feeding experiments: Cultures (5 mL) of *E. coli* transformants harboring pACMod-PAL/C4H + pBADMod2-4CL/CHS, pBADMod2-4CL/CHS, or pBADMod2 alone were grown overnight then inoculated (1:100) into modified M9 medium (50 mL) supplemented with tetracycline and carbenicillin, or with carbenicillin alone. Cultures were induced with arabinose, supplemented with 4-coumaric acid, *trans*-cinnamic acid, caffeic acid, ferulic acid, or 3-(4-hydroxyphenyl)propionic acid (5 mg), and allowed to grow for an additional 24 h before harvest. *E. coli* controls containing the plasmid pBADMod1-4CL or pBADMod1-CHS were tested as described above, with 3-(4-hydroxyphenyl)propionic acid as the supplement.

Growth curves: Cultures (5 mL) of recombinant *E. coli* pACMod-TAL + pBADMod2-4CL/CHS were grown overnight and inoculated (1:200) into modified M9 or TB medium (250 mL) supplemented with chloramphenicol and carbenicillin. These cultures were harvested (10 mL) at induction (taken as the zero time point for production) and samples (10 mL) were removed 12, 24, 36, and 48 h after induction. Samples were centrifuged for 25 minutes at 4000 rpm and 4 °C to remove the cells from the culture medium. Cell pellets were washed once with deionized water and frozen, along with the culture supernatants, at −20 °C prior to extraction.

Extraction conditions: Methanol (5 mL) was added to thawed cell pellets and the mixture placed in a sonicating water bath for 1 h at 4 °C. Cell debris was removed by centrifugation and the methanol was decanted into a fresh conical tube. Water was added to give a final volume of 15 mL. The pH value of the water/methanol mixture was adjusted (to approximately 9.0) to spontaneously convert chalcones into the corresponding flavanones[12] to aid detection and quantification of the products. The mixture was left to stand for 1 h at room temperature then extracted with ethyl acetate (2× 15 mL). The pooled organic phase was frozen at −80 °C for more than 2 h then allowed to warm to room temperature before residual water was removed. The ethyl acetate was removed under vacuum and the product resuspended in acetonitrile (100–200 μL).

The pH value of the culture supernatant (10 mL) was adjusted as described above and the sample incubated at room temperature for 1 h then extracted with ethyl acetate (2×10 mL). The pooled organic phase was frozen and dried as described for the cell pellets. The product was resuspended in acetonitrile (100 μL). All samples were stored at −20 °C prior to HPLC and MS analysis. Extraction of 4-coumaric acid, 3-(4-hydroxyphenyl)propionic acid, and phloretin was conducted as described above but without adjusting the pH value of the culture medium prior to extraction.

HPLC analysis: Pellet and culture supernatant extracts (10 μL) were applied to a Zorbax SB-C18 column (4.6×250 mm, 5 μm; Agilent Technologies, Palo Alto, CA) and eluted with an isocratic mobile phase (water/acetonitrile/acetic acid (69.3:30:0.7); flow rate, 1 mL min^{-1}) by using an Agilent 1100 HPLC system equipped with a photodiode array detector. Compound peaks were identified by comparison of the retention times and UV/Vis spectra of the samples with those of standard compounds. Data obtained by integration of the peaks of known amounts of standard were compared to the peak areas of the unknown compounds for quantification.

LC/ESI-MS and LC/MS/MS: LC-MS was carried out with an LCQ mass spectrophotometer (Thermo Finnigan, USA) equipped with a

Zorbax SB-C18 column. The same elution conditions were used as those applied in the HPLC analysis. Mass fragmentation spectra of standards and the extracted compounds were monitored over a mass range of $m/z = 60–400$ with a negative ESI interface.[29] Parent molecular ions were further fragmented by MS/MS analysis with an ESI interface at the optimal energy for collision-induced dissociation (25–30%). Negative ion data for standard compounds were as follows: 4-coumaric acid, $m/z = 163.1$; *trans*-cinnamic acid, $m/z = 146.9$; naringenin, $m/z = 271.1$; phloretin, $m/z = 273.1$.

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