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ORIGINAL PAPER

Biosynthesis of plant-specific phenylpropanoids by construction of an artificial biosynthetic pathway in *Escherichia coli*

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Abstract Biological synthesis of plant secondary metabolites has attracted increasing attention due to their proven or assumed beneficial properties and health-promoting effects. Phenylpropanoids are the precursors to a range of important plant metabolites such as the secondary metabolites belonging to the flavonoid/stilbenoid class of compounds. In this study, engineered *Escherichia coli* containing artificial phenylpropanoid biosynthetic pathways utilizing tyrosine as the initial precursor were established for production of plant-specific metabolites such as ferulic acid, naringenin, and resveratrol. The construction of the artificial pathway utilized tyrosine ammonia lyase and 4-coumarate 3-hydroxylase from *Saccharothrix espanaensis*, cinnamate/4-coumarate:coenzyme A ligase from *Streptomyces coelicolor*, caffeic acid *O*-methyltransferase and chalcone synthase from *Arabidopsis thaliana*, and stilbene synthase from *Arachis hypogaea*.

Keywords Biosynthesis · Phenylpropanoid · Heterologous expression · Artificial pathway

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Introduction

Phenylpropanoids include several important natural products classes, for example, flavonoids, stilbenoids, lignins, and coumarins. These phenylpropanoid-derived substances have a range of recorded effects on human health, involving antioxidant, antiallergic, antiinflammatory, antithrombotic, and antioncogenic activities [9, 22]. These phenylpropanoid biosynthetic enzymes are, therefore, attractive targets for metabolic engineering and synthetic biology to enhance or initiate the production of economically desirable traits or compounds [7, 10, 23]. Recent advances in the biochemistry of specific enzymes and enzyme complexes have created strategies for the enhancement of phenylpropanoid compounds by modifying pathways [15, 17, 20, 24, 27].

Phenylalanine ammonia-lyase (PAL), which is ubiquitous in plants and fungi, catalyzes nonoxidative deamination of the primary amino acid phenylalanine to cinnamic acid, which is the first reaction of the general phenylpropanoid pathway [26]. Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (C4H) to 4-coumaric acid, which is then activated to 4-coumaroyl-coenzyme A (CoA) by the action of 4-coumarate:CoA ligase (4CL). The activity of 4CL leads to the formation of 4-coumaroyl-CoA, a nodal compound of phenylpropanoid metabolism, which in turn leads to the formation of either flavonoids or stilbenoids. With the action of chalcone synthase (CHS) and three molecules of malonyl-CoA, the cascade enters the pathway of flavonoids, with naringenin being the first and one of the most interesting compounds [1]. The final flavanone structure is formed only when chalcones are stereospecifically isomerized into naringenin by chalcone isomerase (CHI), a reaction known to occur spontaneously in alkaline environments [1]. In an alternative metabolic route, the action of stilbene synthases (STS), with the aid of three molecules of

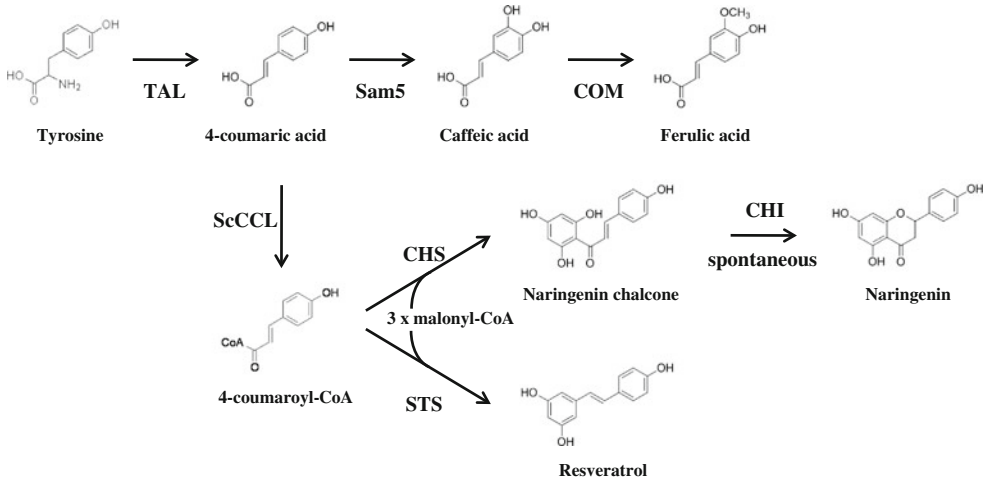

Fig. 1 Engineered biosynthetic pathway in *E. coli*. TAL and Sam5 from *S. espanaensis*, COMT and CHS from *A. thaliana*, ScCCL from *S. coelicolor*, and STS from *A. hypogaea*

malonyl-CoA, guides the cascade to the general stilbenoids pathway with the production of resveratrol (Fig. 1). CHS and STS use a single ketosynthase (KS)-like active site to catalyze the repetitive condensation of acetate units to a CoA-derivatized starter molecule (4-coumaroyl-CoA), typically yielding the mono- and bicyclic aromatic products resveratrol and naringenin, respectively [8, 21].

Our strategy to harness microorganisms for the production of naringenin and resveratrol was to design and express artificial phenylpropanoid pathways with bacterial tyrosine ammonia-lyase (TAL). This was accomplished by assembling TAL from the actinomycete *Saccharothrix espanaensis*, cinnamate/4-coumarate:CoA ligase (ScCCL) from the actinomycete *Streptomyces coelicolor*, CHS from the plant *Arabidopsis thaliana*, and STS from the plant *Arachis hypogaea* on a single plasmid in *Escherichia coli*. In addition, phenylpropioinic acids, such as 4-coumaric acid, caffeic acid, and ferulic acid, can be efficiently produced by recombinant *E. coli* cells expressing the actinomycete TAL, 4-coumarate 3-hydroxylase (Sam5), and plant caffeic acid *O*-methyltransferase (COMT) from *A. thaliana*.

Materials and methods

Strains, plasmids, and chemicals

E. coli DH5α and *E. coli* C41 (DE3) [19] were used for general DNA manipulation and expression of biosynthetic genes, respectively. A pCR®-TOPO vector (Invitrogen, Carlsbad, CA) was used for polymerase chain reaction (PCR) cloning. pET-28a(+) harboring the kanamycin resistance gene was purchased from Novagen (San Diego, CA). 4-Coumaric acid, cinnamic acid, caffeic acid, ferulic acid, naringenin, and resveratrol were purchased from Sigma–Aldrich (St. Louis, MO).

DNA manipulation

Restriction enzymes, Taq DNA polymerase, and other DNA-modifying enzymes were purchased from TaKaRa Biochemicals (Shiga, Japan). DNA ligation was performed using AccuPower® Ligation PreMix and AccuRapid™ DNA ligation kit (Bioneer, Korea). TAL and Sam5 genes from *S. espanaensis* KCTC9392, COMT (GenBank # AY062837) and CHS gene (GenBank # AF112086) from *A. thaliana*, and the ScCCL gene from *S. coelicolor* A3(2) M130 (GenBank # NP628552) were amplified from the genomic DNA or complementary DNA (cDNA) library using the described primer set [1, 3, 14, 25]. The STS gene from *Arachis hypogaea* (GenBank # AB027606) was codon-optimized and synthesized by Codon Devices (Cambridge, MA) [1, 3, 14, 25]. After the DNA manipulation, the absence of undesired alterations during PCR was checked by nucleotide sequencing on an automated nucleotide sequencer.

Construction of pET-T5 M

Each gene expression vector was constructed by standard DNA manipulation including several cycles of fragment-primed PCR. Using genomic DNA and cDNA as a template, three DNA fragments (TAL, Sam5, and COMT) were PCR-amplified with appropriate pairs of primers (Table 1) and cloned into pCR®-TOPO, resulting in pCR-Tal, pCR-Sam5, and pCR-Com, respectively. To construct the pET expression plasmids under the control of the T7 promoter, the *Bgl*II-*Hin*dIII fragment was excised from pCR-Tal and

Table 1 Primers used in this study

Name	Sequence (5′–3′)	Restriction enzyme site
TAL-N	AGATCTACGCAGGTCGTGGAACGT	*Bgl*II
TAL-C	AAGCTTGTGTGCTCATCCGAAATCCTT	*Hin*dIII
Sam5-F	CATATGACCATCACGTCACCTGCGCCGG	*Nde*I
Sam5-R	AAGCTTCAGGTGCCGGGGTTGATCAGGTCGG	*Hin*dIII
COM-F	CATATGGGTTCAACGGCAGAGAC	*Nde*I
COM-R	AAGCTTAGAGCTTCTTGAGTAACTC	*Hin*dIII
CCL-F	CATATGTTCCGCAGCGAGTACGCA	*Nde*I
CCL-R	AAGCTTCATCGCGGCTCCCTGAGC	*Hin*dIII
CHS-N	AGATCTGGTGCTTCTTCTTTGGATGAG	*Nde*I
CHS-C	CTCGAGTTAGAGAGCAACGCTGTG	*Xho*I
kS-R	CTCGAGTTAAATAGCCATCGAGCGCAGGACC	*Xho*I
CPac	TTAATTAATGCGCCGCTACAGGGCGCGTCC	*Pac*I
NPac	TTAATTAATCGCCGCGACAATTTGCGACGG	*Pac*I
Cspe	ACTAGTTCCTCCTTTCAGCAAAAAACCCCTC	*Spe*I
Nspe	ACTAGTAGGTTGAGGCCGTTGAGCACCGCC	*Spe*I

cloned between the *Bam*HI and *Hin*dIII sites of pET-28a(+), resulting in pET-TAL (Fig. 2). The *Nde*I-*Hin*dIII fragment was excised from pCR-Sam5 and cloned between the *Nde*I and *Hin*dIII sites of pET-28a(+), resulting in pET-Sam5 (Fig. 2). The *Nde*I-*Hin*dIII fragment was excised from pCR-Com and cloned between the *Nde*I and *Hin*dIII sites of pET-28a(+), resulting in pET-COM (Fig. 2). To construct an expression vector containing the three genes that were each under the control of the T7 promoter, we first amplified the 1.76-kb DNA fragment containing the TAL coding region using pET-TAL as a template with primer TAL-N and CPac (the sequence is located downstream of the T7 terminator region of the pET vector and contains the designed *Pac*I site; Table 1). Using the pET-Sam5 as a template, the 2.54-kb DNA fragment containing the Sam5 coding region was PCR-amplified with primer NPac (the sequence was located upstream of the T7 promoter region of the pET vector and contained the designed *Pac*I site; Table 1) and CPac. In addition, the 1.88-kb DNA fragment containing the COMT coding region was PCR-amplified using pET-COM as a template with primer NPac and COM-R. The amplified fragments were digested with each restriction enzymes and cloned between *Bam*HI- and *Hin*dIII-digested pET-28a(+) by a four-fragment ligation, resulting in pET-T5 M (Fig. 2). The right direction of Sam5 fragment in pET-T5 M was confirmed by restriction mapping. The gap between the previous terminator and the next promoter (TAL-Sam5 and Sam5-COM) was 696 bp. The pET-T5 M plasmid was orderly constructed, and contained the three genes with their own T7 promoter, ribosome-binding site (RBS), and terminator sequence.

Construction of pET-TLC and pET-TLkS

Using *S. coelicolor* genomic DNA as a template, the ScCCL gene fragment was PCR-amplified with the primer pairs CCL-F and CCL-R (Table 1), and was cloned into pCR®-TOPO, resulting in pCR-CCL. To construct the expression plasmid, the *Nde*I-*Hin*dIII fragment was excised from pCR-CCL and cloned between the *Nde*I and *Hin*dIII sites of pET-28a(+), resulting in pET-CCL (Fig. 2). Using *A. thaliana* cDNA as a template, the CHS gene fragment was PCR-amplified with the primer pairs CHS-N and CHS-C (Table 1), and cloned into pCR®-TOPO, resulting in pCR-CHS. The *Bgl*II-*Xho*I fragment was excised from pCR-CHS and cloned between the *Bam*HI and *Xho*I sites of pET-28a(+), resulting in pET-CHS (Fig. 2). To construct the plasmid containing the artificial naringenin biosynthetic pathway, the previous pET-T5 M cloning method was used. The 2.53-kb DNA fragment containing the ScCCL coding region was PCR-amplified using the pET-CCL as a template with primers NPac and CSpe (the sequence is located downstream of the T7 terminator region of the pET vector and contains the designed *Spe*I site; Table 1). Using pET-CHS as a template, the 1.63-kb DNA fragment containing the CHS coding region was PCR-amplified with primers NSpe (the sequence is located upstream of the T7 promoter region of the pET vector and contains the designed *Spe*I site; Table 1) and CHS-C. The amplified fragments were digested with each restriction enzymes and cloned between *Bam*HI- and *Xho*I-digested pET-28a(+) by a four-fragment ligation, resulting in pET-TLC (Fig. 2). The gap between the previous terminator and the next promoter is 696 bp (TAL-CCL) and 269 bp (CCL-CHS), respectively.

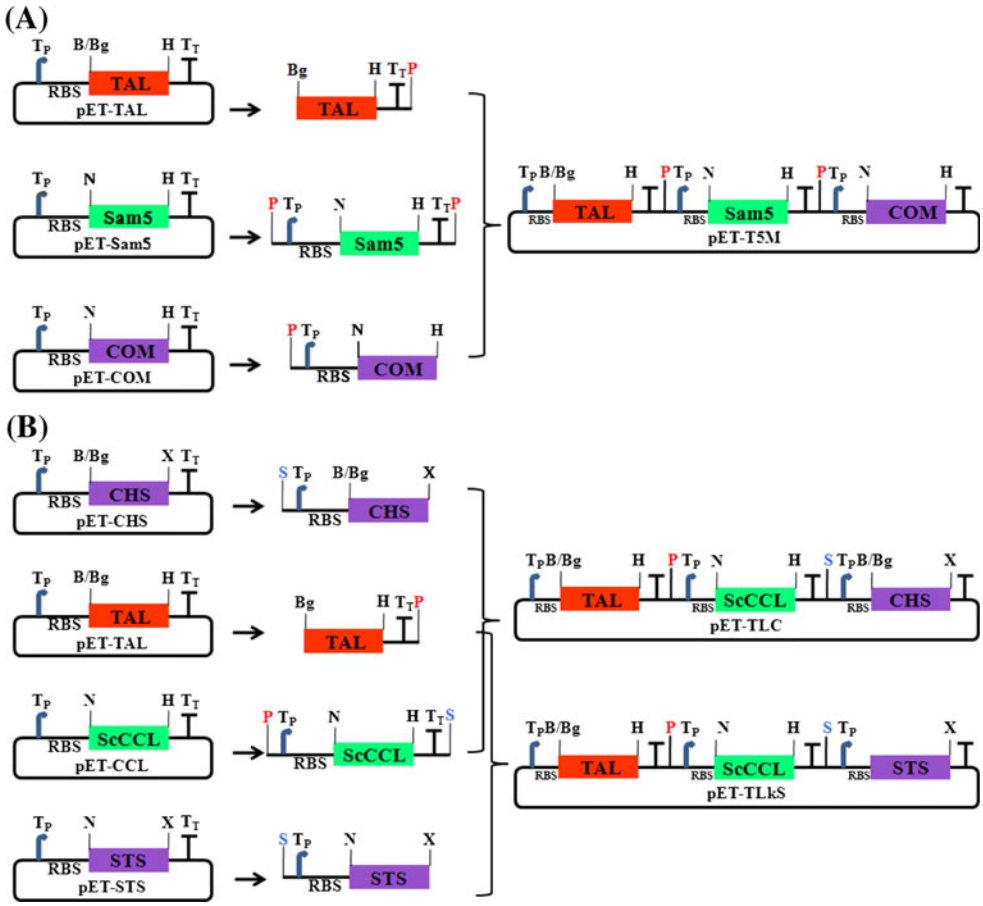

Fig. 2 Organization of the artificial gene clusters used for production of plant-specific compounds in *E. coli*. All three genes contained their own T7 promoter and RBS. Schematic representation of the strategies used for construction of pET-T5 M (**a**), pET-TLC, and pET-TLkS (**b**). All constructs contained the T7 promoter and the RBS in front of each gene, and the T7 terminator located in the rear of each gene. The following abbreviations are used: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; N, *Nde*I; X, *Xho*I; P, *Pac*I; S, *Spe*I; Tp, T7 promoter; T_T, T7 terminator; RBS, ribosomal binding site

In addition, the STS gene of *A. hypogaea* was synthesized and cloned into pUC19. A *Nde*I-*Xho*I fragment of STS (1-kb) was cloned between the corresponding sites of pET-28a(+), resulting in pET-STS (Fig. 2). To construct the plasmid containing the resveratrol biosynthetic pathway, we used the previous cloning method using the *Pac*I and *Spe*I sites. Using pET-STS as a template, the 1.58-kb DNA fragment containing the STS coding region was PCR-amplified with primers NSpe and KS-R. The amplified fragments were digested with each restriction enzyme and cloned between *Bam*HI- and *Xho*I-digested pET-28a(+) by a four-fragment ligation, resulting in pET-TLkS (Fig. 2).

Culture conditions for production

Recombinant *E. coli* C41 (DE3) strains harboring plasmid were precultured overnight at 37°C in Luria–Bertani (LB) medium containing 50 μg/mL kanamycin. The overnight culture was inoculated (1.5%) into fresh LB medium supplemented with the same concentration of kanamycin. The culture was grown at 37°C to optical density at 600 nm (OD_{600}) of 0.6. Isopropyl *β*-D-thiogalactopyranoside (IPTG) was added at final concentration of 1 mM, and the culture was incubated for 5 h. The cells were harvested by centrifugation, suspended, and incubated at 26°C for 36 h in modified M9 medium (M9 medium supplemented with 40 g/L glucose and 25 g/L $CaCO_3$, pH 6.8–7.0) containing 50 μg/mL kanamycin and 1 mM IPTG [21]. At the end of the incubation, the broth cultures had a pH ranging from 5.8 to 6.0. For feeding experiments, cultures were induced with IPTG, supplemented with 4-coumaric acid, cinnamic acid, caffeic acid, and ferulic acid (final concentration, 2 mM), and allowed to grow for an additional 36 h before harvest.

Extraction and analysis of product

Twenty-five milliliters of culture was extracted with an equal volume of ethyl acetate. In particular, the *E. coli* broth cultures containing pET-TLC plasmid were adjusted to pH 9.0 with NaOH [11]. The ethyl acetate was dried under vacuum, and the dried residue was resuspended in 500 μL methanol. Twenty microliters of extract was applied to a J'sphere ODS-H80 column (4.6 × 150 mm i.d., 5 μm; YMC, Kyoto, Japan) using a high-pressure liquid chromatography (HPLC) system [CH_3CN–H_2O (0.05% trifluoroacetic acid) 20:80 to 60:40 over 25 min followed to 100:0 by 1 min, 1 mL/min; Dionex, Sunnyvale, CA, USA] equipped with a photodiode array detector. The retention

times of naringenin and resveratrol were 14.6 and 10.5 min, respectively. Compounds were identified by comparing observed retention time, ultraviolet spectra, and mass chromatogram with those of authentic standards. Liquid chromatography-mass spectrometry (LC–MS) was performed using a Finnigan LCQ mass spectrophotometer (Thermo Electron, Pittsburgh, PA, USA) equipped with an electrospray ionization (ESI) source. HPLC separations were performed using a Finnigan Surveyor HPLC System unit (Thermo Electron) using a hydrosphere C18 column (50 × 2.0 mm, 5 µm; YMC, Kyoto, Japan). The system was operated using a previously described procedure with minor modification [16]. Data-dependent tandem mass spectrometry experiments were controlled using menu-driven software provided with the Xcalibur system. Single-charged radicals for standard compounds were 4-coumaric acid (*m*/*z* 163.1), caffeic acid (*m*/*z* 179.1), ferulic acid (*m*/*z* 193.1), naringenin (*m*/*z* 271.2), and resveratrol (*m*/*z* 227.2).

Results and discussion

Production of 4-coumaric acid, caffeic acid, and ferulic acid by recombinant *E. coli*

To ensure the activity of each of the phenylpropionic acid biosynthetic enzymes, we attempted to clone the recently described TAL and Sam5 from *S. espanaensis* [3], and COMT from *A. thaliana* [5]. *E. coli* containing the pET-TAL plasmid was able to produce 4-coumaric acid (Fig. 3a) but not *trans*-cinnamic acid (the deamination product of phenylalanine) as determined by HPLC. Examination of the TAL protein unambiguously verified tyrosine as its natural substrate. Also, exogenous 4-coumaric acid was fed at induction to recombinant *E. coli* expressing pET-Sam5 grown in modified M9 medium. Caffeic acid was identified by HPLC analysis by comparison with an authentic standard (Fig. 3b). Sam5 catalyzed the conversion of 4-coumaric acid, the TAL reaction product, to caffeic acid. In addition, COMT protein from *A. thaliana*, which is responsible for the biosynthesis of sinapyl alcohol, was partially characterized previously [5]. Presently, the COMT protein was cloned with TAL and Sam5 in *E. coli*. The RBS and/or the T7 promoter were positioned in front of each of the three genes. This construct exhibited the expected effective transcription from the T7 promoter and translation from the ribosome-binding sequence [13]. The distance between the previous terminator and the next promoter (TAL-Sam5 and Sam5-COM) is 696 bp. We think that this distance is sufficient to prevent interference with transcription and the stability of each messenger RNA (mRNA). The strain containing the pET-T5 M plasmid successfully produced ferulic acid as well as caffeic acid, which were detected in all culture supernatants and cell pellets examined, with the majority found in the culture supernatants (Fig. 3c). The retention times of peaks 1, 2, and 3 on HPLC analysis were identical to those of the authentic compounds 4-coumaric acid, caffeic acid, and ferulic acid, respectively. The results

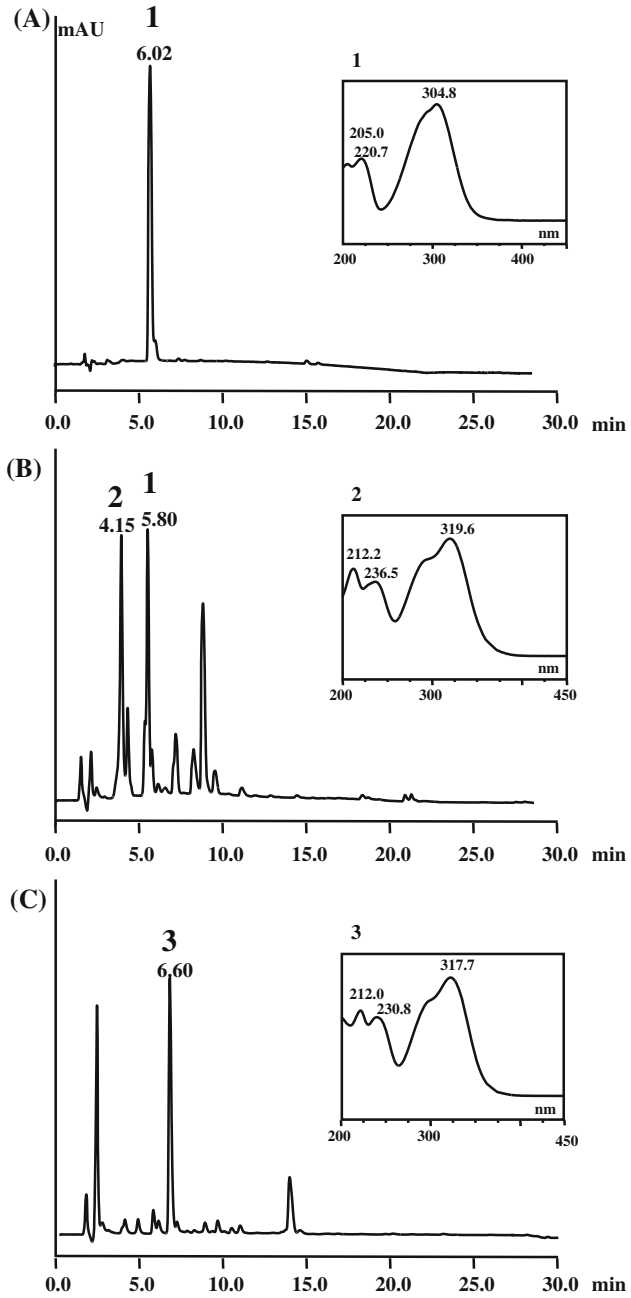

Fig. 3 HPLC analysis of extracts from supernatants of cultures containing *E. coli* cells expressing TAL alone or together with Sam5 and COM. The cells were cultured in modified M9 medium and analyzed after 24 h induction. **a** *E. coli* harboring pET-TAL plasmid. **b** *E. coli* harboring pET-Sam5 fed with 4-coumaric acid. **c** *E. coli* harboring pET-T5 M coexpressing TAL, Sam5, and COM proteins. Absorbance was monitored at 285 nm. The insets show the UV/Vis spectra of the compounds with the indicated HPLC peaks. Peaks indicate 4-coumaric acid (1), caffeic acid (2), and ferulic acid (3)

are consistent with the idea that COMT methylates the C-5 position of the phenolic ring of caffeic acid. The production of ferulic acid using our strategy was 7.1 ± 1.3 mg/L. The heterologous biosynthetic pathway for ferulic acid production can be successfully expressed in the host strain.

Production of naringenin in *E. coli* through an artificial biosynthetic pathway

Naringenin is a gateway compound for a large number of flavonoid-derived plant natural products [9]. To produce naringenin in *E. coli*, an artificial gene cluster containing three genes of heterologous origins (TAL from *S. espanaensis*, ScCCL from *S. coelicolor*, and CHS from *A. thaliana*) was constructed. The use of the bacterial TAL enzyme would bypass the hydroxylation step of C4H for the production of naringenin chalcone from phenylalanine. ScCCL in the Gram-positive filamentous bacterium *S. coelicolor* A3(2) can activate 4-coumaric acid to 4-coumaroyl-CoA, as well as cinnamic acid to cinnamoyl-CoA [14]. CHS is a well-studied ubiquitous plant-specific type III polyketide synthase that catalyzes sequential decarboxylative condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA to produce a new aromatic ring system, naringenin chalcone, the key intermediate in the biosynthesis of flavonoids [1]. Naringenin chalcone was thought to be rapidly converted into naringenin by nonenzymatic isomerization under alkaline conditions. We cloned the CHS gene from *A. thaliana*, and the obtained sequence was not consistent with the published sequences from *A. thaliana* (GenBank # AF112086). However, these nucleotide changes caused silent mutations in the amino acid sequence. Expression of recombinant CHS was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The purified protein displayed the expected molecular weight of near 46.2 kDa (Supplementary Fig. 1). We designed an artificial gene cluster in the pET-TLC plasmid so that tyrosine was converted into naringenin chalcone by the successive actions of TAL, ScCCL, and CHS (Fig. 2). The pET-TLC plasmid consisting of a T7 promoter and RBS upstream of each gene produced the expected efficient transcription and translation of the three genes in *E. coli* [13]. The recombination cells were cultured at 26°C to avoid formation of inclusion bodies of the proteins, since SDS–PAGE analysis of the total proteins showed that, when grown at 37°C, a large amount of 46.2-kDa CHS was evident in both the insoluble and soluble fractions (Supplementary Fig. 1). Even when culturing at 26°C, a considerable amount of the 46.2-kDa CHS was still recovered in the insoluble fraction. On the other hand, no SDS–PAGE-separated proteins with molecular masses of 57.4 and 57.5 kDa, representing TAL and ScCCL, respectively, were detected (Supplementary Fig. 1).

After fermentation, the broth and pellets of cultures grown in modified M9 medium were extracted and analyzed by HPLC. Comparison of the fermentation products of the *E. coli* cells harboring pET-TLC and the vector pET-28a revealed that two new compounds were reproducibly detected in the engineered strain (Fig. 4). The retention times of peaks 1 and 2 were identical to those of authentic 4-coumaric acid and naringenin, respectively (Fig. 4a). We further analyzed these compounds by LC–MS in negative-ion mode. Naringenin was identified by LC–MS (*m/z* 271.26 $[M\text{-}H]^-$) through comparison of the obtained fragmentation pattern with that of an authentic standard and literature data [16]. The production of naringenin using our strategy was 5.3 ± 1.3 mg/L. No pinocembrin compounds as the product of CHS with cinnamic acid were detected in the cell extracts of this strain. This was probably due to the much higher activity of TAL toward tyrosine than toward phenylalanine. However, when cultured with cinnamic acid at final concentration of 2 mM, *E. coli* containing the artificial gene cluster accumulated pinocembrin at *m/z* 255.29 $[M\text{-}H]^-$ (Fig. 4c). This was probably due to the activity of ScCCL and CHS towards cinnamic acid and cinnamoyl-CoA, respectively. In addition, negligible amounts of triketide pyrones (*m/z* 213 and 215 $[M\text{-}H]^-$), a typical derailment product of type III polyketide synthases (PKSs), were detected by the ion chromatograph analysis of LC–MS (Supplementary Fig. 2). However, exogenous supply of caffeic acid and ferulic acid to recombinant *E. coli* did not lead to production of the corresponding flavanones (eriodictyol and homoeriodictyol, respectively). This result confirmed that the ScCCL enzyme could not use caffeic acid and ferulic acid as substrates [14].

Production of resveratrol in *E. coli* through an artificial biosynthetic pathway

Resveratrol, a representative stilbene found in grapes and other food products, has attracted increasing interest due to its inhibitory activity, both in vitro and in vivo, of the initiation, promotion, and progression of carcinogenesis [12]. Several attempts to produce resveratrol by recombinant microorganisms have been reported [2, 25]. Stilbenes are biosynthesized from tyrosine, which are converted to 4-coumaric acid. This acid is then activated to CoA esters, which becomes the substrate of STS, and condenses with three molecules of malonyl-CoA, resulting in resveratrol (Fig. 1).

For the production of resveratrol in microorganisms, the only requirement was to replace the CHS gene in pET-TLC for the naringenin biosynthetic pathway in Fig. 2 with the STS gene. This is one of the advantages of assembling a biosynthetic pathway for a certain product; replacing a single enzyme, the type III PKS, gives a different product, the

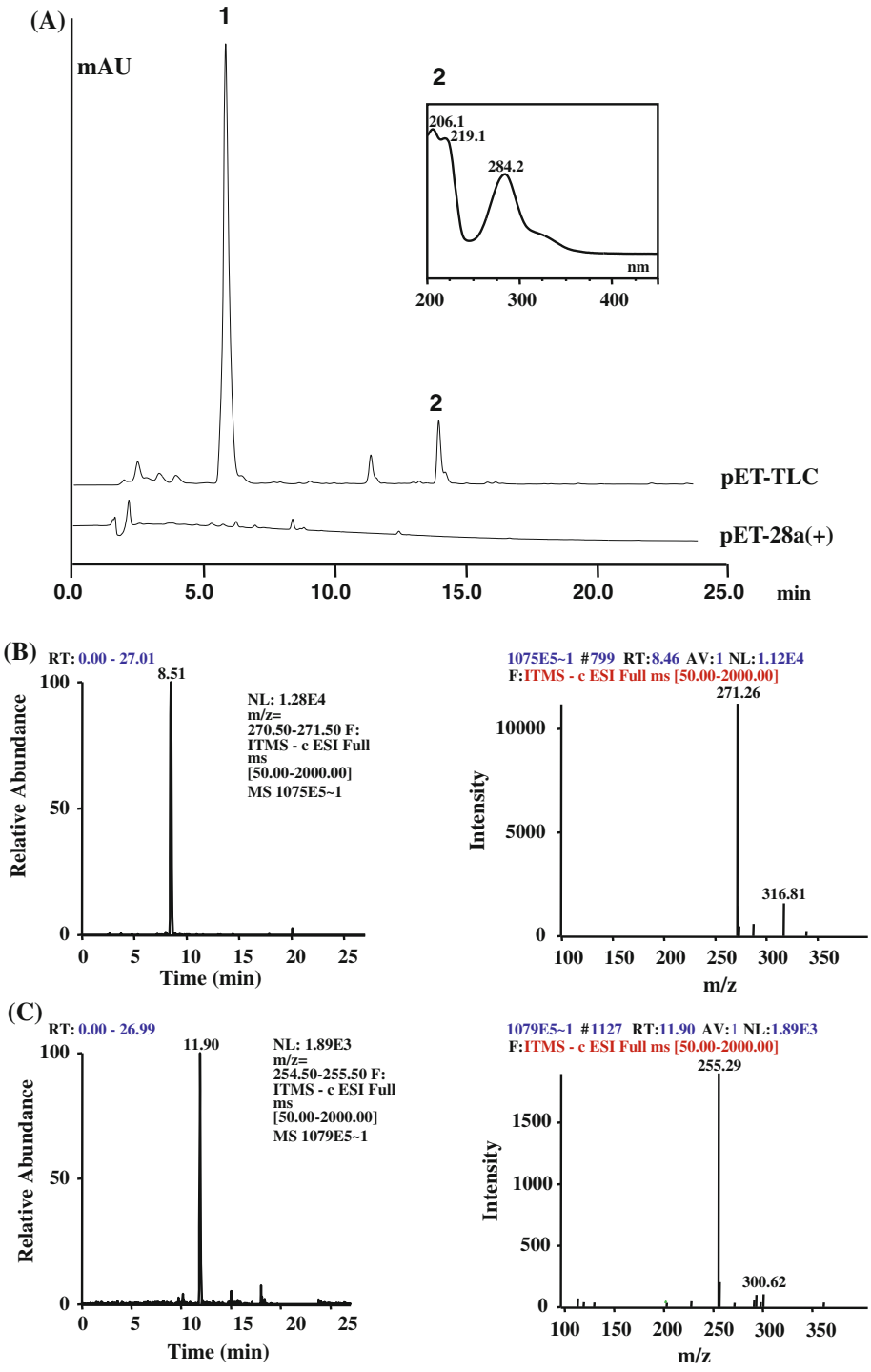

Fig. 4 HPLC and LC–MS analysis of naringenin produced by engineered *E. coli* harboring plasmid pET-TLC. **a** HPLC analysis of extracts from *E. coli* harboring pET-TLC cultures produced naringenin. The insets show the UV/Vis spectra of the naringenin peak. Peaks indicate 4-coumaric acid (1) and naringenin acid (2). **b** Selected ion chromatogram of naringenin (*m/z* 271.26 $[M-H]^-$) produced by *E. coli* harboring pET-TLC. **c** Selected ion chromatogram of pinocembrin at *m/z* 255.29 $[M-H]^-$ produced by *E. coli* harboring pET-TLC. See "Materials and methods" for details of the HPLC and LC–MS protocols

structure of which depends on its catalytic properties, number of extensions, extender substrates employed, and mode of cyclization of the extended intermediate. We first searched for a possible STS in silico and found a candidate in the genome of peanut *A. hypogaea* [25]. The STS gene was synthesized via codon optimization to adapt to its expression host, *E. coli*. Expression of recombinant codon-optimized STS (kSTS) was determined by SDS–PAGE, and the protein showed the expected molecular weight of near 45 kDa (Supplementary Fig. 3). A reduced growth temperature of 26°C may have increased the solubility of the recombinant protein. The culture was extracted with ethyl acetate, and HPLC analysis of the extract revealed the accumulation of a compound with a maximum at 305 nm and elution at 10.5 min in the culture medium (Fig. 5). Both the absorption spectrum and retention time of this

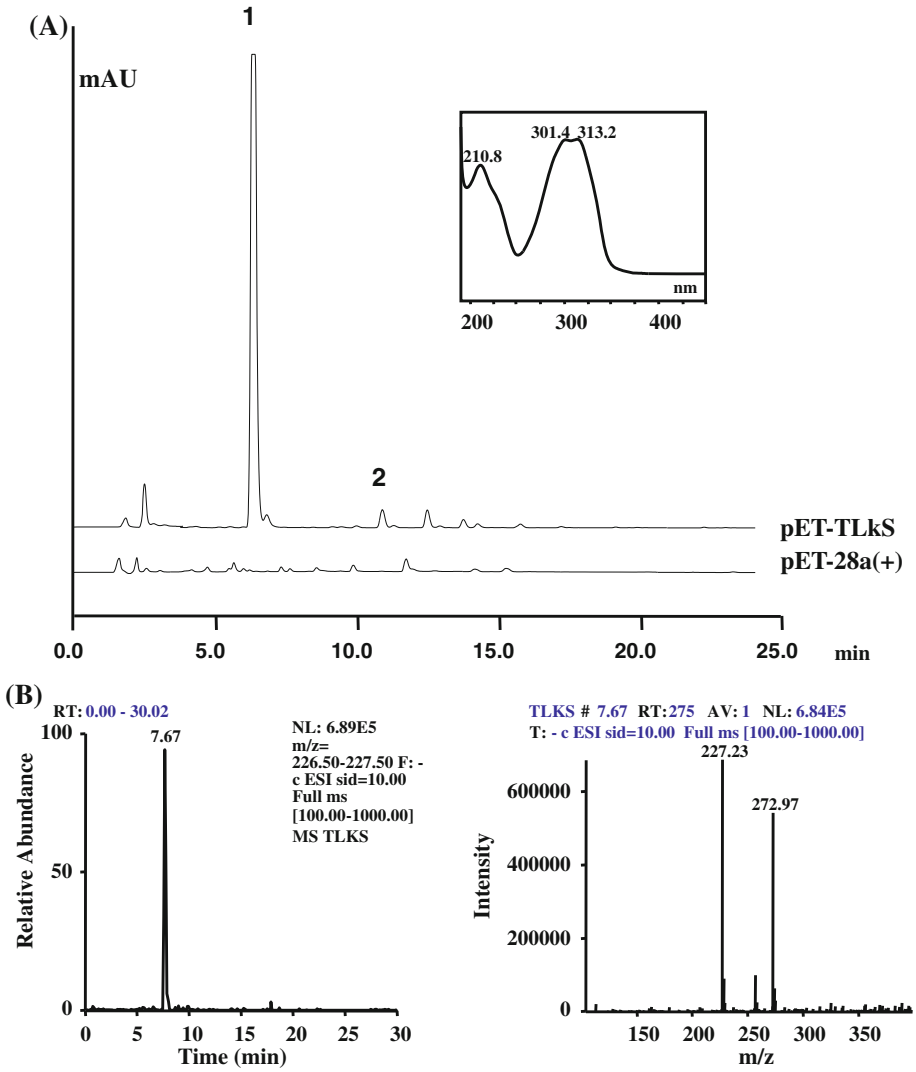

Fig. 5 HPLC and LC–MS analysis of resveratrol produced by engineered *E. coli* harboring pET-TLkS vector. **a** HPLC analysis of extracts from *E. coli* harboring pET-TLkS cultures produced resveratrol. *E. coli* harboring pET28 (empty vector, negative control) did not produce resveratrol. The peak at 6 min represents 4-coumaric acid, and the peak at 10.5 min represents resveratrol. **b** Selected ion chromatogram of resveratrol (*m/z* 227.23 $[M\text{-}H]^-$) produced by *E. coli* harboring pET-TLkS

compound corresponded to those of a standard of resveratrol. Further MS identification revealed that this compound had *m/z* 227.2 $[M\text{-}H]^-$, confirming the identity of the product as resveratrol. The yield of resveratrol was 1.4 ± 0.2 mg/L. Hence, once a pathway for the biosynthesis of precursors is established, the *E. coli* host can be directed toward the synthesis of different phenylpropanoids by varying the choice of the key polyketide synthase.

We have successfully expressed all the biosynthetic genes and engineered the artificial pathway leading to the synthesis of the phenylpropionic acids, naringenin, and resveratrol in *E. coli*, starting from tyrosine. The production of ferulic acid, naringenin, and resveratrol yielded 7.1 ± 1.3 mg/L, 5.3 ± 1.3 mg/L, and 1.4 ± 0.2 mg/L, respectively. It is important to note, however, that the observed phenylpropanoids production was a basal level from the synthetic gene cluster without any metabolic engineering and/or precursor feeding in the fermentation process. However, the yields of phenylpropanoids were very low. We expect that an increase in the amount of malonyl-CoA in *E. coli* under normal culture conditions by overexpression of the acetyl-CoA carboxylase gene would lead to yield enhancement [4, 17, 18]. In addition, use of a tyrosine-fermenting strains would obviate the need to provide amino acids to the culture and elevate the yields of the final product.

To achieve efficient artificial biosynthetic platforms of phenylpropanoids in general, optimizing the functional expression of the biosynthetic enzymes used during the biosynthetic process is a concern. Many of the early studies resorted to more practical approaches of protein expression to improve the effective intercellular concentrations of the functional enzymes in an effort to increase production levels. In this approach, metabolic channeling typically involves the organization of intracellular enzymes so that active sites for consecutive reactions are brought into close proximity. This proximity prevents loss of intermediates by either diffusion or competition with other pathways, and can effectively increase catalytic efficiency by up to two orders of magnitude. In one study, use of synthetic protein

scaffolds recruited metabolic enzymes to an extent that markedly improved the production of mevalonate while lowering the overall cellular metabolic load [6]. Based on an understanding of such organization, we also will try new strategies for efficiently assembling metabolic enzymes into structures that yield high product titers.

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