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Production of Curcuminoids in Engineered *Escherichia coli* S

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Curcumin, a hydrophobic polyphenol derived from the rhizome of the herb *Curcuma longa*, possesses diverse pharmacological properties, including anti-inflammatory, antioxidant, antiproliferative, and antiangiogenic activities. Two curcuminoids (dicinnamoylmethane and bisdemethoxycurcumin) were synthesized from glucose in *Escherichia coli*. *PAL* (phenylalanine ammonia lyase) or *TAL* (tyrosine ammonia lyase), along with *Os4CL* (*p*-coumaroyl-CoA ligase) and *CUS* (curcumin synthase) genes, were introduced into *E. coli,* and each strain produced dicinnamoylmethane or bisdemethoxycurcumin, respectively. In order to increase the production of curcuminoids in *E. coli*, the shikimic acid biosynthesis pathway, which increases the substrates for curcuminoid biosynthesis, was engineered. Using the engineered strains, the production of bisdemethoxycurcumin increased from 0.32 to 4.63 mg/l, and that of dicinnamoylmethane from 1.24 to 6.95 mg/l.

Keywords: Bisdemethoxycurcumin, dicinnamoylmethane, metabolic engineering

Introduction

Substances that have long been used as foods and/or medicines are good sources for natural compounds containing various biological activities. Resveratrol, abundant in wines, is a known anti-aging agent [1]; isoflavonoids, small molecules found majorly in soybean, act as phytoestrogens [2, 3]. Tumeric, which is the major ingredient of curry and is used in cooking as a color additive and preservative, can be found in the rhizome of the herb *Curcuma longa.* It contains curcuminoids such as curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin [4].

Curcuminoids possess diverse biological properties, including anti-inflammatory [5], anticancer [6], antioxidant [7], antiproliferative [8], and antiangiogenic activities [9]. In addition, curcuminoids can affect the prevention and treatment of Alzheimer's disease [10, 11]. Owing to the diverse biological activities of curcuminoids, many derivatives have been synthesized [12, 13].

Curcuminoids are synthesized from hydroxycinnamoyl-CoA thioesters (cinnamoyl-CoA, *p*-coumaroyl CoA, or feruloyl-CoA) and malonyl-CoA by the action of curcuminoid synthase (CUS), which belongs to the type III polyketide synthases (PKSs). Type III PKSs are involved in the synthesis of various phytochemicals, such as flavonoids, stilbenes, and curcuminoids [14, 15]. To date, three genes coding for enzymes that synthesize curcuminoids have been cloned. In *Oryza sativa,* a single enzyme (OsCUS) produced bisdemethoxycurcumin from two molecules of *p*-coumaroyl-CoA and one molecule of malonyl-CoA to make bisdemethoxycurcumin [16]. In *C. longa*, curcuminoids are synthesized by the action of two enzymes, diketide-CoA synthase (DCS) and curcumin synthase (CURS). DCS condenses feruloyl-CoA and malonyl-CoA to synthesize feruloyldiketide-CoA, which is converted into curcumin by CURS [17].

Plant phenolic compounds such as flavonoids, stilbenes, and curcuminoids were produced in *E. coli* by introducing plant and/or microbial genes into the bacterium [18]. These include Type III PKSs such as chalcone synthase and stilbene synthase, which are key enzymes for the synthesis of plant phenolic compounds, as well as 4-cinnamoyl CoA ligase (4CL), which activates hydroxycinnamic acids by converting them into their corresponding CoA thioesters. The aforementioned enzymes (*i.e.*, typeIII PKSs together with 4CL) can be used for flavonoid, stilbene, and

curcuminoid production from hydroxycinnamic acids. These can be combined with enzymes that synthesize hydroxycinnamic acids from either tyrosine or phenylalanine, such as tyrosine ammonia lyase (TAL) and phenylalanine ammonia lyase (PAL). TAL converts tyrosine into *p*-coumaric acid (a precursor of bisdemethoxycurcumin), whereas PAL converts phenylalanine into cinnamic acid (a precursor of dicinnamoylmethane). However, depending on the organism of origin, both enzymes may display substrate ambiguity; in addition to their preferred substrates and reactions, PAL may also possess TAL activity and, similarly, TAL may also display PAL activity. Katsuyama *et al.* [17] used PAL from *Rhodotorula rubra*. Because of this enzyme's substrate ambiguity, supplementation of the engineered *E. coli* with any of these two amino acids leads to the production of a mixture of three curcuminoids.

In this study, we synthesized specific curcuminoids, mainly bisdemethoxycurcumin and dicinnamoylmethane, by introducing genes coding for tyrosine-specific TAL and phenylalanine-specific PAL, respectively. In addition, introducing both genes resulted in both curcuminoids.

Materials and Methods

Construction of *E. coli* Expression Vector

PAL (AtPAL) was cloned from *Arabidopsis thaliana* [19], *TAL* (SeTAL) from *Saccharothrix espanaensis* [20], and *4CL* (Os4CL) from *O. sativa* [21], whereas pA-tyrA-aroG-SeTAL was constructed previously [20].

Two constructs containing both *Os4CL* and *OsCUS* were made. In the first construct, both genes were controlled by independent T7 promoters. *Os4CL* was cloned into the EcoRI/NotI site of the pETDuet-1 vector (Merck Millipore, USA). *OsCUS* cDNA of *O. sativa* (*OsCUS*; AK109558) was cloned by RT-PCR as described previously [22] using the following primers: forward 5′-ATCATATGGCACCGACGACGACCATG-3′ (nucleotide of the added NdeI site are underlined); reverse, 5′-TTAATTCACATG AGAGGTGGCGTGCAA-3′. The resulting *OsCUS* was digested with NdeI and subcloned into the second cloning site (NdeI/EcoRV) of the Os4CL-harboring pCDFDuet-1 vector (Merck Millipore). In the second construct, two genes were controlled by the same T7 promoter. In order to create it, *OsCUS* was first amplified with the following primers: forward, 5′-atGAATTCG ATGGCACCGACGACGACCAT-3′ (the added EcoRI site is underlined.); reverse 5′-atACTAGTTTAATTCACATGAGAGGTGG-3′ (the added SpeI site is underlined.). *Os4CL* was amplified with added SpeI site and NotI sites as well as a ribosome binding sequence using the following primers: forward, 5′-ATACTA GTaggaggattacaaaATGGATCCGA-3' (nucleotides of the ribosome binding site are written in lower case; nucleotides of the SpeI site are underlined.); reverse 5′-ATGCGGCCGCTTAGCTGCTTTTGG GCGC-3′ (the added NotI site is underlined.). The resulting PCR products were digested with SpeI and ligated. It was amplified using the *OsCUS* forward primer and the *Os4CL* reverse primer. The resulting PCR product was digested with EcoRI/NotI and subcloned between the corresponding sites of the pCDF-1 Duet vector.

The two *E. coli* strains, namely B-T (*tyrR* deletion mutant) and B-TP (*tyrR/pheA* deletion mutant), were previously created [20]. Strain B-TT was made from B-T using the Quick and Easy Conditional Knockout Kit (Gene Bridges, Germany). First, the kanamycin cassette in the B-T strain was removed with a 708-FLPe expression plasmid coding for FLPe recombinase. The removal of the kanamycin cassette was verified by colony PCR. Using this mutant as a host, the *tyrA* gene was replaced by the tyrA FRT-PGK-gb2-neo-FRT cassette.

Strain B-TAL was created as follows; *aroG* and *tyrA* were cloned into the pACYDuet vector (Merck Millipore) using EcoRI/SalI and NdeI/KpnI, respectively. Subsequently, the XhoI site in *aroG* was silenced using the following primers: forward, 5′-AGGCAA TCAGAGCCTGGAGAGCGGGGAGCCG-3′; reverse, 5′-CGGCTC CCCGCTCTCCAGGCTCTGATTGCCT-3′. The resulting plasmid was called p-T1. The FRT-PGK-gb2-neo-FRT cassette was amplified using the following primers: forward, 5′-ATctcgagAATTAACCCT CACTAAAGGGCG-3′ (lower case letters indicate the XhoI site); reverse, 5′-ATcctaggTCGAGGAAGTTCCTATACTTTC-3′ (lower case letters indicate the AvrII site). Afterwards, the cassette was cloned into the corresponding site of p-T1. This new plasmid was named p-T2 and served as a template for the generation of the ΔtyrRaroG-tyrA-FRT-PGK-gb2-neo-FRT cassette, using the following primers: forward, 5′-GATGCACGGTTACGATGCGCCCATCTA CACCAACGTGACCTATCCCATTACGGTgctatcatgccataccgcga-3′; reverse, 5′-AATTGCGGCCTATATGGATGTTGGAACCGTAAGA GAAATAGACAGGCGGTccgtgtgcttctcaaatgcc-3′ (upper and lower case letters indicated sequences from the lacZYA operon and the pACYCDuet vector, respectively). The PCR product was gel-purified and used in transformation according to Kim *et al.* [20]. The positive colonies were first screened using LB medium containing 50 μg/ml kanamycin. Afterwards, colony PCR was used to verify the clones positive for mutant lacZYA with the following primers: forward, 5′-CGTCGTCCCCTCAAACTGGC-3′; reverse, 5′-CACCGATTGTTACCGTGTAGTCAT3′. This strain was named B-T1. A 708-FLPe plasmid encoding for FLPe recombinase was used to remove the kanamycin cassette from B-T1. Removal of the kanamycin cassette was verified by colony PCR. The new strain was named B-T2. In order to integrate *aroG*, *SeTAL*, and *tyrA* into the *tyrR* loci of B-T2, the aroG-SeTAL-tyrA-coding part of p-T1 was amplified with the following primers: forward, 5′-GTTGACAGAAACCTTCCTGCTATCCAAATAGTGTCATATCA TCATATTAATTGTTCTTTTTTCAGGTGAAGGTTCCCATGgctat catgccataccgcga-3′; reverse, 5′-TTGCACCATCAGGCATATTCG CGCTTACTCTTCGTTCTTCTTCTGACTCActgatgtccggcggtgcttt-3′ (the upper and lower case letters are used for sequences from *tyrR* and the pACYCDuet vector, respectively). The PCR product was

Table 1. Plasmids and strains used in the present study.

Plasmids or *E. coli* strain	Relevant properties or genetic marker	Source or reference
Plasmids		
pACYCDuet	P15A ori, Cm^r	Novagen
pCDFDuet	CloDE13 ori, Str^r	Novagen
pETDuet	f1 ori, Amp^r	Novagen
pGEX 5X-3	pBR322ori, Amp^r	GE Healthcare
pA-AtPAL	pACYCDuet harboring *PAL* from *Arabidopsis thaliana*	[20]
pA-SeTAL	pACYCDuet harboring *TAL* from *Saccharothrix espanaensis*	[19]
pA-aroG-tyrA-SeTAL	pACYCDuet harboring *aroG* and *tyrA* from *E. coli*, and *TAL* from *S. espanaensis*	[20]
pE-pOsCUS-pOs4CL	pETDuet harboring *CUS* and *4CL* from *Oryza sativa*; each gene controlled by an independent T7 promoter	This study
pE-pOsCUS-Os4CL	pETDuet harboring *CUS* and *4CL* from *O. sativa*; both genes controlled by one T7 promoter	This study
BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm lon* (DE3)	
B-T	BL21(DE3) *ΔtyrR*	[20]
B-TP	BL21(DE3) *ΔtyrR/ΔpheA*	[20]
B-TT	BL21(DE3) *ΔtyrR/ΔtyrA*	This study
B-TAL	BL21(DE3) integrating aroG-SeTAL-tyrA into *tyrR* and aroG-tyrA into LacZYA of *E. coli*	This study
B-CU1	BL21 harboring pA-SeTAL, and pE-pOsCUS-pOs4CL	This study
B-CU2	BL21 harboring pA-SeTAL and pE-pOsCUS-Os4CL	This study
B-CU3	B-T harboring pA-SeTAL and pE-pOsCUS-Os4CL	This study
B-CU4	B-TP harboring pA-SeTAL and pE-pOsCUS-Os4CL	This study
B-CU5	B-TP harboring pA-aroG-SeTAL-tyrA and pE-pOsCUS-Os4CL	This study
B-CU6	BL21 harboring pA-AtPAL and pE-pOsCUS-Os4CL	This study
B-CU7	B-T harboring pA-AtPAL and pE-pOsCUS-Os4CL	This study
B-CU8	B-TT harboring pA-AtPAL and pE-pOsCUS-Os4CL	This study
B-CU9	B-TAL harboring pC-AtPAL and pE-pOsCUS-Os4CL	This study
B-CU10	B-TAL harboring pA-SeTAL, pC-AtPAL, and pE-pOsCUS-Os4CL	This study

gel-purified and transformed according to Kim *et al*. [20]. The positive colonies were screened first using LB medium containing 50 μg/ml chloramphenicol, followed by colony PCR to verify the clones positive for the *tyrR* mutation with the following primers: forward, 5′-CGTCAGCTTATCGGTCAGGTG-3′; reverse, 5′-CAG CCAGCTGGTGGATGAAA-3′

Both the plasmid and the *E. coli* strains that were used in the current study are summarized in Table 1.

Culture Condition and Analysis of Reaction Products

The transformants were grown in LB medium containing 50 μg/ml antibiotics at 37°C for 18 h. One-tenth of the culture was inoculated into new medium and grown at 37°C for 7 h. Cells were harvested and resuspended to $OD_{600} = 1.0$ in M9 medium containing 1% glucose, 1% yeast extract, 50 μg/ml antibiotics, and 1 mM IPTG. After 24 h at 37°C, the culture supernatant was collected, adjusted to pH 3.0 using 5 N HCl, and extracted with ethyl acetate. The ethyl acetate layer was collected after centrifugation and dried out in a Speed Vac at 45°C. The pellet was dissolved in 60 μl of dimethyl sulfoxide (DMSO) and analyzed using a high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, USA) equipped with a photodiode array detector, on a Varian polar C_{18} reversed-phase column (4.60 × 250 mm, 0.45 μm; Varian, USA). Analytical scale chromatography was used with a mobile phase consisting of H_2O with 0.1% formic acid (pH 3.0) and acetonitrile. The percentage of acetonitrile in the mobile phase was programmed as follows: 20% at 0 min, 45% at 10 min, 80% at 20 min, 100% at 20.10 min, 100% at 25 min, 20% at 25.10 min, and 20% at 30 min. The flow rate was 1 ml/min. The elutant was detected at 280, 320, and 420 nm.

Dicinnamoylmethane and bisdemethoxycurcumin, which were used as standards in HPLC, were not available commercially; they were synthesized by and obtained from Professor Chong's laboratory at Konkuk University [23]. The quantification of curcuminoids was carried out with HPLC using diferuloylmethane (Sigma-Aldrich, USA) as a standard. Diferuloylmethane was dissolved in DMSO at the concentration of 100 μM and used as a stock solution. The stock was diluted with M9 medium to concentrations ranging from 1 to 100 μM (0.36 to 36.34 mg/l). Analysis of the diluted solutions confirmed the linearity between the concentration of diferuloulmethane and absorbance (measured in mAU) in HPLC. The means and standard errors were calculated from triplicate experiments.

Results and Discussion

Synthesis of Bisdemethoxycurcumin

Curcuminoids are condensation products of various hydroxycinnamoyl-CoA thioesters with malonyl-CoA. The structure of the final product varies depending on the type of hydroxycinnamoyl-CoA thioester used. For the biosynthesis of diverse curcumins, *PAL*, *TAL*, *4CL*, and *CUS* genes are needed. The enzymatic characterization of the protein encoded by each gene has been done [16, 21, 24, 25]. Based on these results, we designed a pathway for the synthesis of bisdemethoxycurcumin, dicinnamoylmethane, or cinnamoyl *p*-coumaroylmethane. Initially, we decided to synthesize bisdemethoxycurcumin, which is produced from *p*-coumaric acid. *p*-Coumaric acid, in turn, is synthesized from tyrosine by the action of TAL, and is turned to *p*-coumaroyl-CoA by Os4CL. OsCUS is specific for *p*-coumaroyl-CoA and cinnamoyl-CoA [16] (Fig. 1). Three genes, *SeTAL*, *Os4CL*, and *OsCUS*, were cloned into *E. coli* expression vectors. *Os4CL* and *OsCUS* were subcloned into one vector. Two constructs were constructed; the first (pC-pOsCUS-pOs4CL) had a pseudo-operon form; that is, the two genes (*Os4CL* and *OsCUS*) were controlled by two

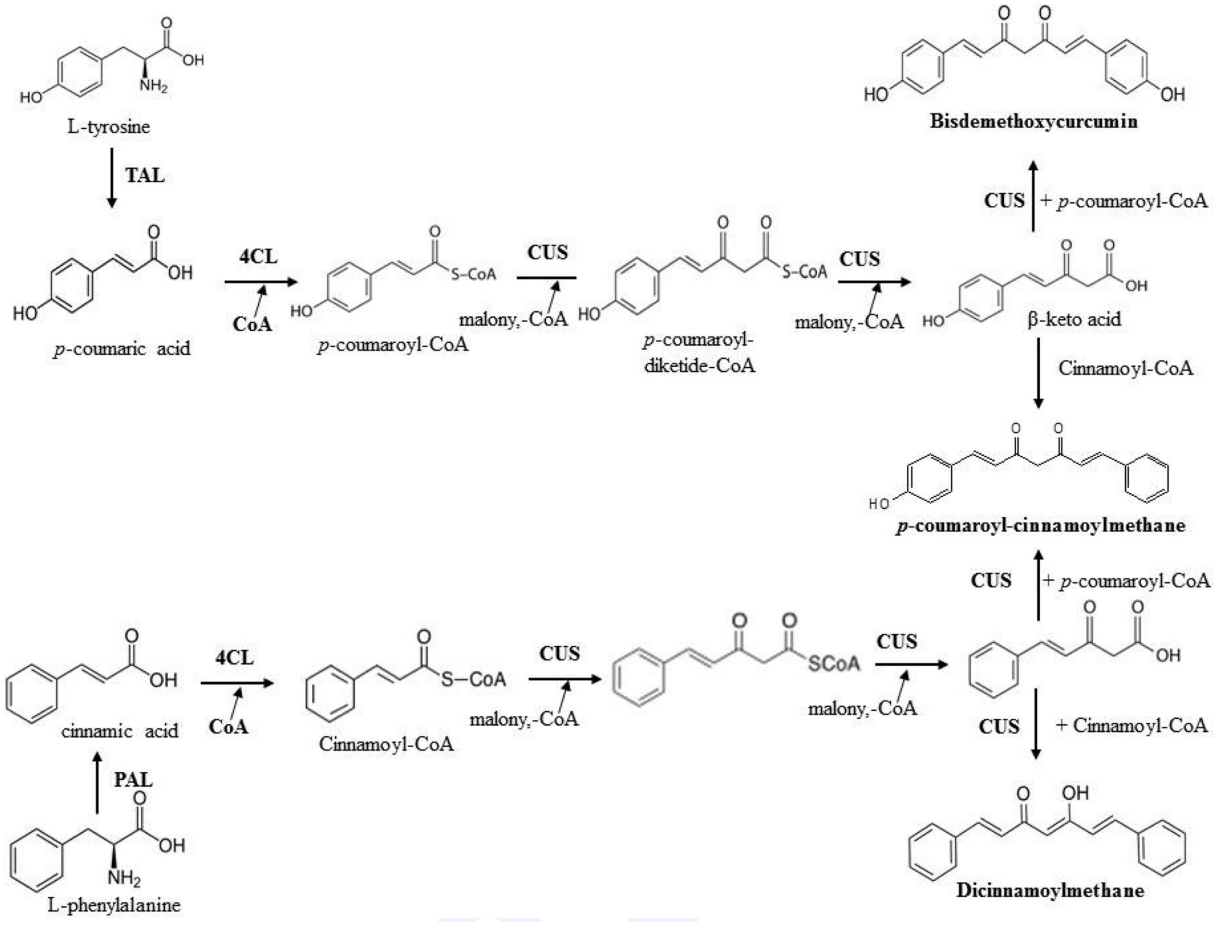

Fig. 1. Biosynthesis pathway of three curcuminoids in *E. coli*.
TAL, tyrosine ammonia lyase; 4CL, 4-cinnamate coenzyme A ligase; CUS, curcuminoid synthase; PAL, phenylalanine ammonia lyase.

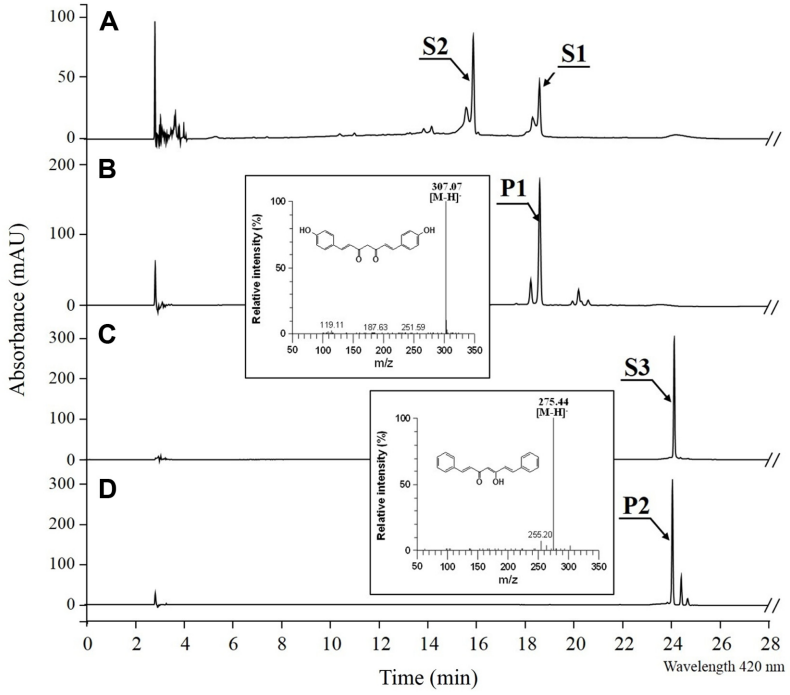

Fig. 2. HPLC analysis of the reaction products.
(**A**) Standard bisdemethoxycurcumin. S1, enol-form of bisdemethoxycrucumin; S2, keto-form of bisdemethoxycurcumin. (**B**) HPLC analysis of reaction product from strain B-CU2. Insert is the MS spectrum of the reaction product in a negative mode. (**C**) Standard dicinnamoylmethane (S1). (**D**) HPLC analysis of reaction products from strain B-CU6. Insert is the MS spectrum of the reaction product in a negative mode.

distinct promoters. The second one (pC-pOsCUS-Os4CL) was an operon-type construct; that is, the two genes were regulated by the same promoter. Each construct, along with pA-SeTAL, was transformed into *E. coli* BL21 (DE3), and the resulting transformants (B-CU1 and B-CU2) were tested for the production of bisdemethoxycurcumin. A new compound was contained in the culture filtrate of B-CU1 and B-CU2, whose retention time was equal to that of the bisdemethoxycurcumin standard (Figs. 2A and 2B). In addition, the molecular mass of the reaction product was 308.07 Da (Fig. 2), which matched the predicted molecular mass of bisdemethoxycurcumin. These results suggested that both strains synthesized bisdemethoxycurcumin. The *E. coli* that was transformed with the operon-type construct (B-CU2) produced 0.32 mg/l bisdemethoxycurcumin, whereas that transformed with the pseudo-operon-type construct (B-CU1) produced only 0.09 mg/l. Therefore, we decided to choose the operon-type construct for the rest of the study.

It has been shown that TAL and PAL are inhibited by cinnamic acid and cinnamoyl-CoA [26, 27]. Therefore, Os4CL and OsCUS, which are located downstream of TAL and PAL in bisdemethoxycurcumin biosynthesis, were cloned in a higher copy number plasmid than those used for *AtPAL* or *SeTAL*, in order to avoid the accumulation of cinnamic acid or *p*-coumaric acid.

As tyrosine is a substrate of TAL, its supply is critical for the production of bisdemethoxycurcumin. Two *E. coli* strains, B-CU3 and B-CU4, were used to test the productivity of bisdemethoxycurcumin. B-CU3 was derived from the B-T strain, which is a *tyrR* deletion mutant, whereas B-CU4 was from the B-TP strain, which is a *tyrR*/*pheA* deletion mutant. Deletion of these genes in *E. coli* has been shown to increase the production of tyrosine, and subsequently, flavonoids [26, 28]. Three genes (*SeTAL*, *Os4CL*, and *OsCUS*) were transformed into B-T and B-TP, and the resulting B-T and B-TP transformants were given the names B-CU3 and B-CU4, respectively. The respective bisdemethoxycurcumin productivity values in B-CU3 and B-CU4 were approximately 2.68 mg/l and 4.63 mg/l, respectively, both of which were much higher than that of B-CU2 (0.32 mg/l) (Fig. 3).

We also tested the effect of tyrosine on the production of bisdemethoxycurcumin by overexpressing *aroG* (2-dehydro-3-deoxyphosphoheptonate aldolase) and *tyrA* (chorismate mutase/prephenate dehydrogenase). Overexpression of both genes has been shown to increase the production of

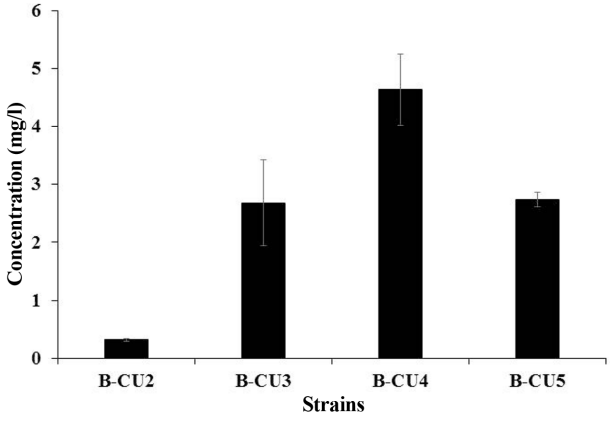

Fig. 3. Production of bisdemethoxycurcumin in various *E. coli* mutant strains. Each *E. coli* strain (B-CU2 to B-CU5) produced different amounts of tyrosine.

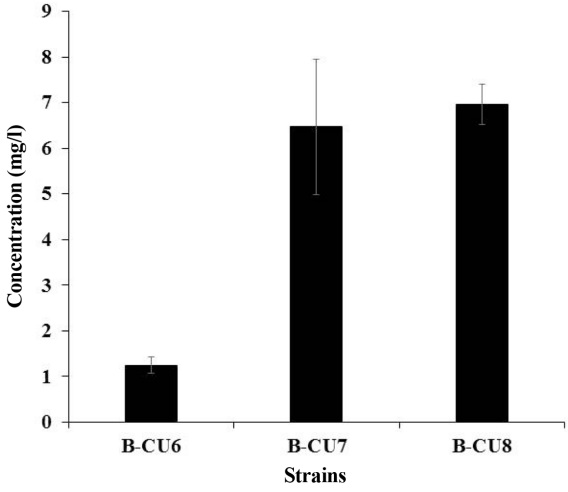

Fig. 4. Production of dicinnamoylmethane in *E. coli* strains. B-CU6, a wild type; B-CU7, *tyrR* deletion mutant; B-CU8, *tyrR* and *tyrA* deletion mutant. Each strain was transformed with pA-AtPAL and pE-pOsCUS-4CL.

tyrosine [29]. The B-CU4 strain was chosen for the *aroG*/*tyrA* overexpression experiment. The resulting strain was named B-CU5 and it produced approximately 2.74 mg/l bisdemethoxycurcumin, which is 40% less than the productivity of B-CU4 (Fig. 3). It is worth mentioning that the B-CU10 strain, which contains only two copies of *aroG* and *aroA* (one integrated into the *tyrR* locus and the other into *LacXYZ*), produced approximately 6.02 mg/l bisdemethoxycurcumin. Thus, although B-CU10 was used to synthesize three curcuminoids (bisdemethoxycurcumin, cinnamoyl *p*-coumaroyl methane, and dicinnamoylmethane; See below), it had produced more bisdemethoxycurcumin than B-CU3 and B-CU4. These results indicated that the metabolic balance between tyrosine and other substrates such as malonyl-CoA is tuned accurately to maximize the production of bisdemethoxycurcumin.

Synthesis of Dicinnamoylmethane

AtPAL instead of SeTAL was used for the synthesis of dicinnamoylmethane. AtPAL specifically converts phenylalanine to cinnamic acid, which is then used to create dicinnamoylmethane [19]. *Os4CL* and *OsCUS* were expressed using the operon-type construct because it was more effective than the pseudo-operon-type construct during the synthesis of bisdemethoxycurcumin. We transformed *E. coli* BL21 (DE3) with pA-AtPAL and pE-pOsCUS-Os4CL; the resulting transformant (B-CU6) was examined for the production of dicinnamoylmethane. As shown in Fig. 2D, the reaction product displayed the same retention time as the authentic dicinnamoylmethane standard and the molecular mass of the reaction product was 276.44 Da, which matched with that of dicinnamoylmethane. In order to increase the production of dicinnamoylmethane, two more strains, B-CU7 and B-CU8, were made. Strain B-CU7 was derived from a *tyrR* deletion mutant, whereas B-CU8 was deleted for *tyrA* and *tyrR* to increase the supply of phenylalanine. The production of dicinnamoylmethane was increased from 1.24 mg/l in B-CU6 to 6.47 mg/l in B-CU7 and 6.95 mg/l in B-CU8. This indicated that the phenylalanine abundance was a critical factor for determining the final yield of dicinnamoylmethane (Fig. 4). Production of bisdemethoxycurcumin was not observed in B-CU6, B-CU7, and B-CU8, confirming that AtPAL is specific for phenylalanine.

Synthesis of Dicinnamoylmethane, Cinnamoyl *p*-Coumaroylmethane, and Bisdemethoxycurcumin in the Same *E. coli* strain

Dicinnamoylmethane is synthesized from phenylalanine, whereas bisdemethoxycurcumin is produced from tyrosine. As AtPAL and SeTAL are specific for phenylalanine and tyrosine, respectively, we hypothesized that coexpression of these two genes in *E. coli* would result in the synthesis of dicinnamoylmethane, cinnamoyl *p*-coumaroylmethane, and bisdemethoxycurcumin. To test this hypothesis, the *SeTAL* gene was first integrated into the *tyrR* locus of *E. coli*. The resulting strain (B-TAL) contained one copy of *SeTAL* in its chromosome. Strain B-CU9 was created by transforming B-TAL with pC-AtPAL and pE-pOsCUS-Os4CL, and was used to identify the synthesized product(s). The results are shown in Fig. 5A. Dicinnamoylmethane, derived from

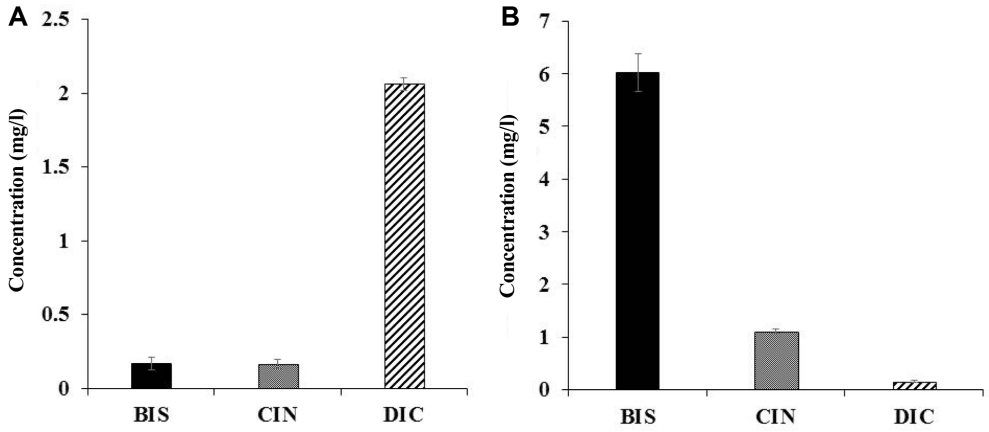

Fig. 5. Cosynthesis of different curcuminoids within different *E. coli* strains.
(**A**) Synthesis of three different curcuminoids in strain B-CU9, which contains a copy of the *SeTAL* gene in the chromosome and multiple copies of *AtPAL* in plasmids. As a result, dicinnamoylmethane, which is produced from phenylalanine by the action of AtPAL, was the major product. (**B**) Synthesis of curcuminoids in strain B-CU10. The amount of synthesized bisdemethoxycurcumin was higher than that of dicinnamoylmethane. BIS, bisdemethoxycurcumin; CIN, cinnamoyl *p*-coumaroylmethane; DIC, dicinnamoylmethane.

cinnamic acid that was produced from phenylalanine by the action of AtPAL, was a major product (2.06 mg/l), whereas bisdemethoxycurcumin, derived from *p*-coumaric acid that was produced from tyrosine by the action of SeTAL, was synthesized as a minor product (0.17 mg/l). Cinnamoyl *p*-coumaroylmethane, derived from the products of both enzymes, was another minor product (0.16 mg/l) (Fig. 5A). The production of cinnamoyl *p*-coumaroylmethane was confirmed by MS (Fig. S1). These results suggested that the amount of *p*-coumaric and cinnamic acids produced in strain B-CU9 was linked to both the type and the amounts of the products. The fact that dicinnamoylmethane was the major product may be attributed to the lower copy number of SeTAL compared with AtPAL, resulting in cinnamic acid being produced in a higher amount compared with *p*-coumaric acid. In order to test this hypothesis, B-CU9 was transformed with pA-SeTAL. The resulting strain (B-CU10) displayed productivity values of 0.16, 6.02, and 1.11 mg/l for dicinnamoylmethane, bisdemethoxycurcumin, and cinnamoyl *p*-coumaroylmethane, respectively (Fig. 5B), indicating that the *p*-coumaric acid/cinnamic acid ratio determined the type of curcuminoids produced.

In summary, we synthesized three different curcuminoids using OsCUS. A previous study has shown that OsCUS can use cinnamoyl-CoA and *p*-coumaroyl-CoA, but not feruloyl-CoA [16]. CURS and DCS from *C. longa* prefers feruloyl-CoA to *p*-coumaroyl-CoA or caffeoyl-CoA [30]. Thus, it is important to select the appropriate enzyme for a specific curcuminoid.

In this study, we achieved production of 4.63 mg/l bisdemethoxycurcumin and 6.95 mg/l dicinnamoylmethane. Rodrigues *et al*. [30] achieved production of 0.3 and 0.2 mg/l bisdemethoxycurcumin from either *p*-coumaric acid or tyrosine, respectively, whereas 70 mg/l curcumin was synthesized from ferulic acid. We concluded that our system is optimized for the synthesis of bisdemethoxycurcumin and dicinnamoylmethane.

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