Appl Biol Chem (2016) 59(2):173–179
DOI 10.1007/s13765-015-0137-4

Online ISSN 2468-0842
Print ISSN 2468-0834

ARTICLE

Bacterial synthesis of four hydroxycinnamic acids

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Received: 1 October 2015 / Accepted: 16 October 2015 / Published online: 19 January 2016

Abstract Hydroxycinnamates are a class of phenolic compounds that have a C6-C3 carbon backbone. Hydroxycinnamic acids are derived from cinnamic acid via hydroxylation or methylation and are found in foods such as pears, coffee beans, and dandelions. They are involved in protection against chemotherapy side effects and the prevention of cardiovascular disease and cancer. We synthesized four types of hydroxycinnamates (*p*-coumaric acid, cinnamic acid, caffeic acid, and ferulic acid) from glucose in *Escherichia coli* by introducing different combination of four genes: tyrosine ammonia lyase, phenylalanine ammonia lyase, *S.espanaensis* monooxygenase, and *Oryza sativa O*-methyltransferase. The final yields of hydroxycinnamic acids were increased by engineering the metabolic pathway of *E. coli*. Using these strategies, 100.1 mg/L *p*-coumaric acid, 138.2 mg/L caffeic acid, 64 mg/L ferulic acid, and 1072.3 mg/L cinnamic acid were synthesized.

Keywords Hydroxycinnamic acid · Phenylalanine ammonia lyase · Tyrosine ammonia lyase

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Introduction

Plants synthesize diverse phenolic compounds via phenylpropanoid pathway using phenylalanine (Dixon and Paiva 1995; Vogt 2010). A key enzyme that converts phenylalanine into cinnamic acid is phenylalanine ammonia lyase (PAL). Cinnamic acid becomes *p*-coumaroyl-coenzyme A (CoA) via hydroxylation and CoA attachment. *p*-Coumaroyl-CoA serves as a starting compound for the synthesis of benzoic acids, stilbenes, aurones, coumarins, flavonoids, and lignins. Cinnamic acid and *p*-coumaric acid along with caffeic acid, ferulic acid, and sinapinic acid are classified as hydroxycinnamic acids (HCs) (Vogt 2010). HCs have potential as precursors for the synthesis of thermoplastics, cosmetics, and flavors (Kaneko et al. 2006; Sariaslani 2007). They have diverse biological activities, including antiviral, antidiabetic, anticancer, and antiplatelet activities (Huang et al. 1988; Forkmann and Martens 2001; Jung et al. 2006; Luceri et al. 2007).

Although plants are major HC producer, the extraction of HCs from plant materials is not feasible due to the complexity and inefficiency of the separation and purification processes. Therefore, alternative approaches have been examined (Rodriguez et al. 2014). Microorganisms, particularly *Escherichia coli*, are good systems to synthesize phenolic compounds including HCs (Wang et al. 2015). To synthesize HCs in *E. coli*, phenylalanine and tyrosine serve as substrates. PAL converts phenylalanine into cinnamic acid and tyrosine ammonia lyase (TAL) converts tyrosine into *p*-coumaric acid. Caffeic acid is synthesized from *p*-coumaric acid via monooxygenase, Sam5 from *Saccharothrix espanaensis*, and further *O*-methylation by *O*-methyltransferase (OMT) results in the synthesis of ferulic acid. In this report, we used a combination of four genes, *PAL*, *TAL*, *Sam5*, and *OMT*, to

synthesize four HCs, cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid, in *E. coli*. In addition, we used *E. coli* mutants in which genes in the shikimate pathway were deleted to increase phenylalanine and/or tyrosine. Using these strategies, 100.37 mg/L *p*-coumaric acid, 1332.84 mg/L cinnamic acid, 168.3 mg/L caffeic acid, and 73.3 mg/L ferulic acid were synthesized in *E. coli*.

Materials and methods

Constructs

PAL (AT3G53260) was cloned from *Arabidopsis thaliana* by reverse transcription-polymerase chain reaction (RT-PCR). mRNA was isolated from 2-week-old A. *thaliana* whole plants using the Plant Total RNA Isolation Kit (Qiagen, Hilden, Germany). cDNA was synthesized using oligo dT primers and Omniscript reverse transcriptase (Qiagen). PCR was carried out using Hotstart Taq DNA polymerase (Qiagen) and 5′-ATGAATTCGATGGATCAAATCGAAGCAATG-3′ and 5′-ATGCGGCCGCTTAGCAAATCGGAATCGGAG-3′ as primers (EcoRI and NotI sites are underlined). The resulting product was subcloned into the EcoRI/NotI sites of pACYCDuet1 and named pA-AtPAL. *TAL* (Kim et al. 2013), *Sam5* (Lee et al. 2014), and *ROMT9* (Kim et al. 2006) were cloned previously. pA-SeTAL, pA-aroG-SeTAL-tyrA, and pA-aroG^{fbr}-SeTAL-tyrA^{fbr} were also cloned previously (Kim et al. 2013).

Construction of the *SeTAL* integrated *E. coli* strain

To integrate *SeTAL* into *tyrR* of *E. coli* chromosome, *SeTAL* was cloned into a pACYCDuet vector using EcoRI/HindIII site with primers (SeTAL-F and SeTAL-R) listed in Table 1. The resulting construct was used to generate a transformation cassette by PCR for a *tyrR* deletion with two primers (TyrR-del-F and TyrR-del-R), which include the region from the T7 promoter to the chloramphenicol resistance gene region and the *tyrR* region. The PCR product was purified and used to integrate *SeTAL* into *tyrR* with the Quick and Easy Conditional Knockout Kit (Gene Bridges, Heidelberg, Germany). The resulting *E. coli* strain contained *SeTAL*, which is controlled by the T7 promoter, and chloramphenicol resistance gene (Cm^R) at the *tyrR* locus of the chromosome. The tyrR-SeTAL strain contained only one copy of SeTAL. The selection marker for the tyrR-SeTAL strain was chloramphenicol.

Culture conditions and analysis of the reaction product

The transformants were grown in LB medium containing 50 μg/mL antibiotics for 18 h at 37 °C. The culture was inoculated onto fresh LB medium containing antibiotics and grown until $OD_{600} = 1.0$. The cells were harvested and resuspended in M9 medium containing 2 % glucose, 1 % yeast extract, 50 μg/mL antibiotics, and 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). The culture was grown for 24 h at 30 °C. The culture medium was extracted with ethyl acetate, and the upper layer was dried out in a Speed Vac and dissolved in 60 μL of DMSO (dimethyl sulfoxide). The product was analyzed via high-performance liquid chromatography (HPLC) (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a photodiode array detector using a Polar C18 reversed-phase column (Agilent, Santa Clara, CA, USA; 4.60 × 250 mm, 0.45 μm). The HPLC conditions were described in Yang et al. (2015). The standard cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Results and discussion

Synthesis of cinnamic acid in *E. coli*

Cinnamic acid is synthesized from phenylalanine by PAL. *PAL* was cloned from *A. thaliana* and transformed into *E. coli* BL21 (DE3). The resulting transformant B-CiA1 was examined for the production of cinnamic acid. As shown in Fig. 1A, B-CiA1 produced a compound that had a same retention and spectrum as those of a standard cinnamic acid. This result showed that cinnamic acid was successfully synthesized in the B-CiAl strain.

The phenylalanine content could influence the production of cinnamic acid. Therefore, we used *E. coli* mutants with deletions of gene(s) in the shikimate pathway. The B-T strain in which *tyrR* was deleted and the B-TT strain in which *tyrR* and *tyrA* were deleted were used to test the effect of phenylalanine on the production of cinnamic acid. TyrA competes with PheA for prephenate. Therefore, the deletion of *tyrA* resulted in increased phenylalanine production (Lütke-Eversloh and Stephanopoulos 2007; Juminaga et al. 2012). We made two strains by transforming B-T and B-TT with pA-SeTAL. The production of cinnamic acid ranged from 686.4 mg/L in B-CiA1 to 971.5 mg/L in B-CiA2 and 1002.6 mg/L in B-CiA3. Higher phenylalanine production was associated with higher cinnamic acid production (Fig. 2). Using B-CiA3, approximately 1072.3 mg/L of cinnamic acid could be synthesized in 36 h (Fig. 3A).

Synthesis of *p*-coumaric acid, caffeic acid, and ferulic acid in *E. coli*

p-Coumaric acid is synthesized from tyrosine by TAL. The *TAL* gene was cloned from *S. espanaensis*. TAL derived

Table 1 Plasmids, *Escherichia coli* strains, and primers used in this study

Plasmids or *E. coli* strain or primers	Relevant properties or genetic marker	Source or reference
Plasmids		
pACYCDDuet	P15A ori, Cm^r	Novagen
pCDFDuet	CloDE13 ori, Str^r	Novagen
pA-SeTAL	pACYCDuet carrying *TAL* from *Saccharothrix espanaensis*	Kim et al. 2013
pC-SeTAL	pCDFDuet carrying *TAL* from *Saccharothrix espanaensis*	
pA-AtPAL	pACYCDuet carrying *PAL* from *Arabidopsis thaliana*	
pC-Sam5	pCDFDuet carrying monooxygenase (Sam5) from *Saccharothrix espanaensis*	Lee et al. 2014
pC-Sam5-ROMT5	pCDFDuet carrying monooxygenase (Sam5) from *Saccharothrix espanaensis* and O-methyltransferase (ROMT9) from *Oryza sativa*	
pA-aroG-SeTAL-tyrA	pACYCDuet carrying *SeTAL*, *aroG,* and *tyrA*	Kim et al. 2013
pA-aroG^{fbr}-SeTAL-tyrA^{fbr}	pACYCDuet carrying *SeTAL* and feedback inhibition free of *aroG* and *tyrA*	Kim et al. 2013
Strains		
BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm lon* (DE3)	
B-T	BL21(DE3) *FRT-ΔtyrR::FRT-kan^R-FRT*	Kim et al. 2013
B-TP	BL21(DE3) *ΔtyrR::FRT- ΔPheA::FRT-kan^R-FRT*	Kim et al. 2013
B-TT	BL21(DE3) *ΔtyrR::FRT- ΔTyrA::FRT-kan^R-FRT*	Kim et al. 2013
B-pCA1	BL21 (DE3) in which SeTAL was integrating into *tyrR* of *E. coli*	This study
B-pCA2	B-T harboring pA-SeTAL	This study
B-pCA3	B-T harboring pC-SeTAL	This study
B-pCA4	B-TP harboring pA-SeTAL	This study
B-pCA5	B-TP harboring pA-aorG-SeTAL-TyrA	This study
B-pCA6	B-TP harboring pA-aorG^{fbr}-SeTAL-TyrA^{fbr}	This study
B-CA1	B-TP harboring pA-aorG^{fbr}-SeTAL-TyrA^{fbr} and pC-Sam5	This study
B-FA1	B-TP harboring pA-aorG^{fbr}-SeTAL-TyrA^{fbr} and pC-Sam5-ROMT5	This study
B-CiA1	BL21 (DE3) harboring pA-AtPAL	This study
B-CiA2	B-T harboring pA-AtPAL	This study
B-CiA3	B-TT harboring pA-AtPAL	This study
Primers	Sequence (5′ → 3′)	
SeTAL-F	ATgaattcGATGACGCAGGTCGTGGAACGT	
SeTAL-R	CATAAGCTTTCATCCGAAATCCTTCCCGTC	
TyrR-del-F	GTTGACAGAAACCTTCCTGCTATCCAAATAG TGTCATATCATCATATTAATTGTTCTTTT TTCAGGTGAAGGTTCCCATGgctatcatgccataccgcga	
TyrR-del-R	TGAAAGCATAATTTAATATGCCTGATGGTGT TGCACCATCAGGCATATTCGCGCTTACTCT TCGTTCTTCTTCTGACTCAccgtgtgcttctcaaatgcc	

from plants showed the activity toward both tyrosine and phenylalanine (Rosler et al. 1997). However, an in vitro enzyme analysis with TAL from *S. espanaensis* showed that it used tyrosine preferentially over phenylalanine (Berner et al. 2006). We tested whether *E. coli* harboring *TAL* from *S. espanaensis* produced only *p*-coumaric acid. The B-pCA1 strain was grown, and the production of *p*-coumaric acid was examined by HPLC. As shown in Fig. 1, culture filtrates from B-pCA1 showed a peak that had a similar retention time and spectrum to those of *p*-coumaric acid (Fig. 1B, H), and the molecular mass of the reaction product was 164 Da, which corresponded with that of *p*-coumaric acid (data not shown). We could not observe a product that showed a similar retention time with cinnamic acid, even when we used other strains such as B-pCA2 and B-pCA3 (see below).

We tested the effects of *TAL* copy number on the production of *p*-coumaric acid. B-pCA1 had a single copy of *TAL* on the chromosome, which is regulated by the T7 promoter. B-pCA2 and B-pCA3 contained multiple copies of

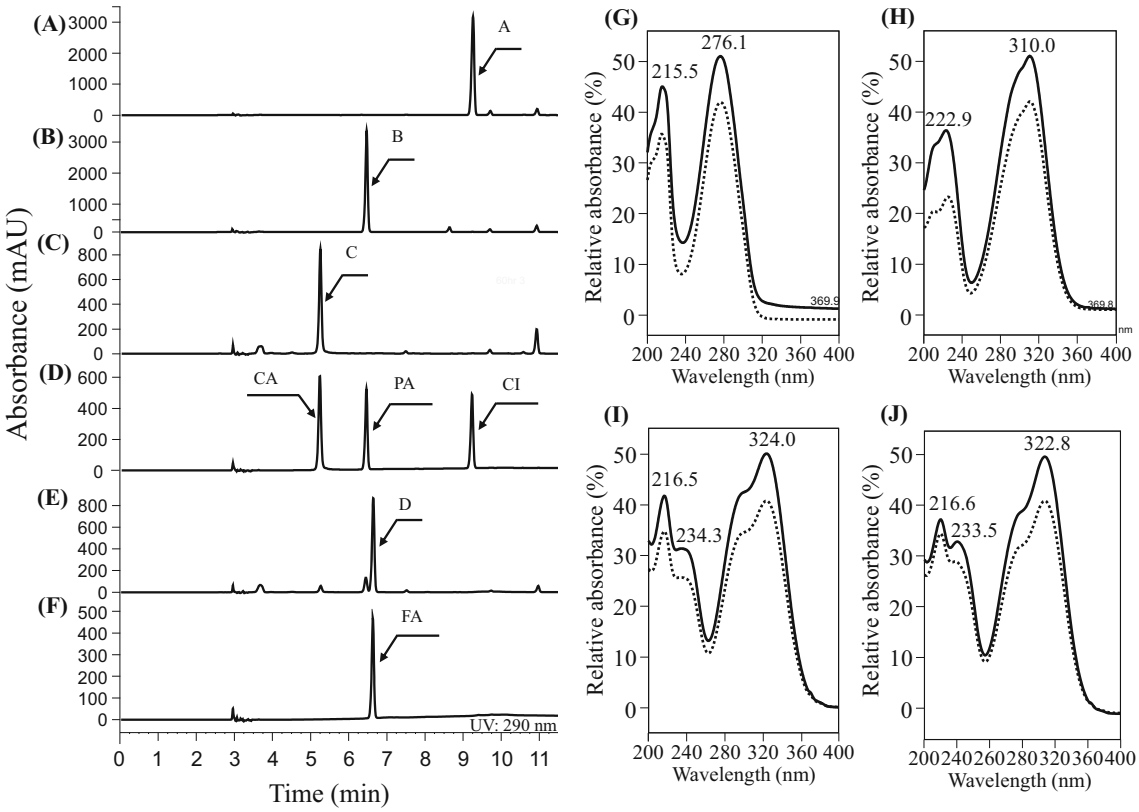

Fig. 1 Analysis of the reaction products using high-performance liquid chromatography (HPLC). (**A**) Product from B-CiA1 as indicated A; (**B**) product from B-pCA1 as indicated B; (**C**) product from B-CA1 as indicated C; (**D**) standards (caffeic acid [CA], *p*-coumaric acid [PA], and cinnamic acid [CI], in order); (**E**) product from B-FA1 as indicated D; (**F**) ferulic acid standard (FA); (**G**) spectra for peak A (*dotted line*) and cinnamic acid (*straight line*); (**H**) spectra for peak B (*dot line*) and *p*-coumaric acid (*straight line*); (**I**) spectra for peak C (*dotted line*) and caffeic acid (*straight line*); (**J**) spectra for peak D (*dotted line*) and ferulic acid (*straight line*)

TAL as plasmids. B-pCA2 contained 10–12 because *TAL* was cloned in the pACYC-Duet1 vector (Tolia and Joshua-Tor. 2006). B-pCA3 has 20–40 copies of *TAL*. However, B-pCA2 produced more *p*-coumaric acid (44.2 mg/L) than both B-pCA1 (4.8 mg/L) and B-pCA3 (40.5 mg/L) (Fig. 4A). For up to ten copies, the *TAL* copy number was directly related to the yield of *p*-coumaric acid under our experimental conditions. However, more greater than 10 copies could result in a metabolic burden to the cell and therefore lead to reduced production of *p*-coumaric acid. The PCA-2 strain showed the highest production of *p*-coumaric acid.

We tested whether the tyrosine content is related to the production of *p*-coumaric acid. We used three strains (the *tyrR* deletion mutant [B-T], and the *tyrR* and *pheA* double deletion mutant [B-TP]), and *TAL* was transformed into each strain. *TyrR* is a transcription factor in the shikimate pathway and is inhibited by the endproduct tyrosine. Accordingly, the deletion of *tyrR* appears to increase tyrosine production. PheA directed prephenate to phenylalanine production. The production of *p*-coumaric acid in each transformant (B-pCA2 and B-pCA4) was examined. As expected, B-pCA4 (84.5 mg/L) showed higher production of *p*-coumaric acid than B-pCA2 (44.2 mg/L). This suggested that higher tyrosine production resulted in higher *p*-coumaric acid production. We overexpressed genes in the shikimate pathway to increase the tyrosine content. Wild-type versions and feedback inhibition versions of *aroG* and *tyrA* were used. Two new strains, B-pCA5 and B-pCA6, were generated, and the ability to produce *p*-coumaric acid was tested in each strain. The production of *p*-coumaric acid was higher in B-pCA5 (96.7 mg/L) than in B-pCA4 (84.5 mg/L). However, strain B-pCA6 (82.7 mg/L) produced a similar amount of *p*-coumaric acid as B-pCA4 (Fig. 4B). Although the tyrosine content is directly correlated to the production of *p*-coumaric acid, the metabolic balance between tyrosine and other cellular metabolites is also critical to increase the yield of *p*-coumaric acid. Using B-pCA5, the production of *p*-coumaric acid was monitored. The production of *p*-coumaric acid continued to increase until 36 h, which points the maximum production was reached (100.1 mg/L = 60.9 μM), and then production decreased slightly.

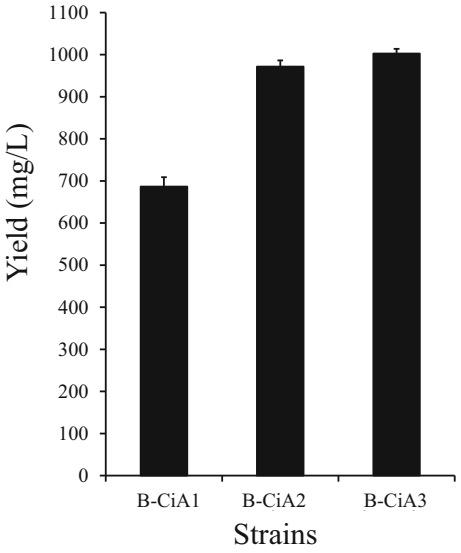

Fig. 2 Effect of mutant *Escherichia coli* on the production of cinnamic acid

Caffeic acid is synthesized from *p*-coumaric acid by hydroxylation at the carbon 3 position. Sam5 hydroxylates several phenolic compounds, including *p*-coumaric acid (Berner et al. 2006; Lee et al. 2014). We transformed strain B-pCA5 with pC-Sam5, and the resulting strain, B-CA1, was used for the production of caffeic acid. As shown in Fig. 1C, B-CA1 produced a product whose retention time in the HPLC analysis was indistinguishable from that of standard caffeic acid. This suggested that Sam5 functioned properly and caffeic acid was synthesized in *E. coli*. Using the B-CA1 strain, approximately 138.2 mg/L (76.7 μM) caffeic acid was synthesized after 36 h (Fig. 3B).

To synthesize ferulic acid, an additional gene, *ROMT9*, was needed. ROMT9 is an O-methyltransferase; it can transfer a methyl group from *S*-adenosyl methionine (SAM) to caffeic acid and flavonoids (Kim et al. 2006). ROMT9 was subcloned into the second cloning site of pC-Sam5, and the resulting construct (pC-Sam5-ROMT9) was transformed into B-pCA5. The resulting strain, B-FA1, was tested for the production of ferulic acid. Based on an HPLC analysis of the reaction product, the peak had the same retention time and spectrum as those of standard ferulic acid (Fig. 1E, J). This showed that ferulic acid was successfully synthesized. B-pCA5 synthesized approximately 64 mg/L (33.0 μM) ferulic acid after 36 h (Fig. 3B).

We synthesized *p*-coumaric acid, caffeic acid, and ferulic acid without adding tyrosine to the medium. It is possible that *E. coli* used tyrosine derived from the yeast extract. Yeast extract contains 0.8 % free tyrosine according to BD Bionutrients Technical Manual (BD Bioscience, San Jose, CA, USA). This means that 80 mg/L (approximately 440 μM) tyrosine was present in the medium. Considering cellular metabolism, this amount of tyrosine is not sufficient to synthesize *p*-coumaric acid, caffeic acid, or ferulic acid; it is necessary to produce tyrosine using glucose via shikimate pathway of *E. coli*. Therefore, in our study, the production *p*-coumaric acid in *E. coli* was correlated with the amount of tyrosine.

We synthesized four HCs, cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid. Each HC or two or three HCs have been synthesized and reported by other groups

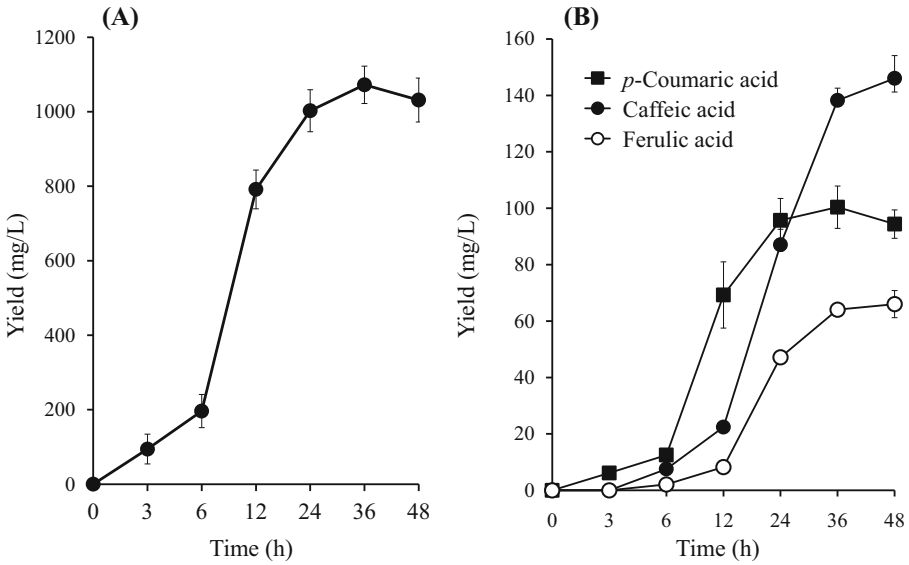

Fig. 3 Production of cinnamic acid (**A**), *p*-coumaric acid, caffeic acid, and ferulic acid (**B**) using engineered *E. coli*

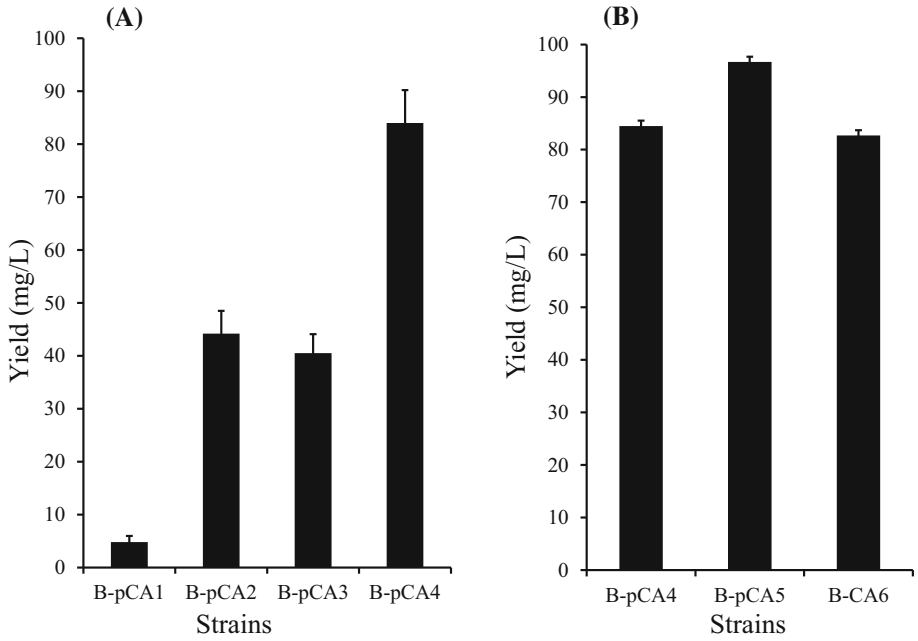

Fig. 4 Effect of *TAL* copy number and construct (**A**) and mutant *E. coli* (**B**) on the production of *p*-coumaric acid

(Furuya et al. 2012; Kang et al. 2012; Vargas-Tah et al. 2015; Zhang and Stephanopoulos 2013). The biosynthesis of all four HCs has not reported to date. The final HC yields reported by other groups are difficult to compare owing to differences in units and culture media. The bioconversion of *p*-coumaric acid to caffeic acid using *E. coli* harboring cytochrome P450 showed the highest yield (2.8 g/L) (Furuya et al. 2012). The yield of caffeic acid rages from 106 mg/L (Zhang and Stephanopoulos 2013) to 150 mg/L (Kang et al. 2012). Therefore, the caffeic acid yield observed in the current study (138.2 mg/L) was comparable to that in previous studies.

The final yield of cinnamic acid was much higher than that of *p*-coumaric acid, caffeic acid, and ferulic acid. The content of free phenylalanine in the yeast extract is 2.0 %, which is 2.5-fold higher than that of free tyrosine (0.8 %). However, this cannot fully account for the final yield because approximately 200 μg/mL phenylalanine was present in the medium. *E. coli* should synthesize more phenylalanine than tyrosine in order to synthesize more cinnamic acid than *p*-coumaric acid. Difference in PAL and TAL activity could explain the observed difference in the final yield of *p*-coumaric acid and caffeic acid. Previous studies have attempted to optimize PAL or TAL in order to increase the final yields (Vargaz-Tah et al. 2015). These two enzymes are responsible for the conversion of phenylalanine and tyrosine to *p*-coumaric acid and caffeic acid, respectively. Additionally, the shikimate pathway involved in the biosynthesis of tyrosine and phenylalanine is conserved until prephenate (Rodriguez et al. 2014). PheA is responsible for the synthesis of phenylpyruvate from prephenate. TyrA functions in the synthesis of tyrosine. The activity of pheA might be much higher than that of tyrA, which leads to increased phenylalanine synthesis.

Acknowledgments This work was supported by a grant from the Next-Generation BioGreen 21 Program (PJ00948301), the Rural Development Administration, and Priority Research Centers Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2009-0093824).

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