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Production of hydroxycinnamoyl-shikimates and chlorogenic acid in *Escherichia coli*: production of hydroxycinnamic acid conjugates

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Abstract

Background: Hydroxycinnamates (HCs) are mainly produced in plants. Caffeic acid (CA), *p*-coumaric acid (PA), ferulic acid (FA) and sinapic acid (SA) are members of the HC family. The consumption of HC by human might prevent cardiovascular disease and some types of cancer. The solubility of HCs is increased through thioester conjugation to various compounds such as quinic acid, shikimic acid, malic acid, anthranilic acid, and glycerol. Although hydroxycinnamate conjugates can be obtained from diverse plant sources such as coffee, tomato, potato, apple, and sweet potato, some parts of the world have limited availability to these compounds. Thus, there is growing interest in producing HC conjugates as nutraceutical supplements.

Results: Hydroxycinnamoyl transferases (HCTs) including hydroxycinnamate-CoA shikimate transferase (HST) and hydroxycinnamate-CoA quinate transferase (HQT) were co-expressed with 4-coumarateCoA:ligase (4CL) in *Escherichia coli* cultured in media supplemented with HCs. Two hydroxycinnamoyl conjugates, *p*-coumaroyl shikimates and chlorogenic acid, were thereby synthesized. Total 29.1 mg/L of four different *p*-coumaroyl shikimates (3-*p*-coumaroyl shikimate, 4-*p*-coumaroyl shikimate, 3,4-di-*p*-coumaroyl shikimate, 3,5-di-*p*-coumaroyl shikimate, and 4,5-di-*p*-coumaroyl shikimate) was obtained and 16 mg/L of chlorogenic acid was synthesized in the wild type *E. coli* strain. To increase the concentration of endogenous acceptor substrates such as shikimate and quinate, the shikimate pathway in *E. coli* was engineered. A *E. coli aroL* and *aroK* gene were mutated and the resulting mutants were used for the production of *p*-coumaroyl shikimate. An *E. coli aroD* mutant was used for the production of chlorogenic acid. We also optimized the vector and cell concentration optimization.

Conclusions: To produce *p*-coumaroyl-shikimates and chlorogenic acid in *E. coli*, several *E. coli* mutants (an *aroD* mutant for chlorogenic acid production; an *aroL*, *aroK*, and *aroKL* mutant for *p*-coumaroyl-shikimates production) were made and each mutant was tested using an optimized construct. Using this strategy, we produced 235 mg/L of *p*-coumaroyl-shikimates and 450 mg/L of chlorogenic acid.

Keywords: Chlorogenic acid, Hydroxycinnamic acid, Hydroxycinnamate-CoA quinate transferase, Hydroxycinnamate-CoA shikimate transferase

Background

Hydroxycinnamates (HCs) such as caffeic acid (CA), *p*-coumaric acid (PA), ferulic acid (FA) and sinapic acid (SA) are mainly produced in plants. Because HCs are anti-oxidants that can scavenge free radicals, it has been suggested that consumption of HCs might prevent cardiovascular disease and some types of cancer. In

addition, several biological functions of HCs have been described, such as protection against side effects of chemotherapy and anti-osteoclast activity [1].

In plants, HCs are usually conjugated with other compounds such as quinic acid, shikimic acid, malic acid, anthranilic acid, and glycerol [2]. Chlorogenic acid, the conjugate of CA and quinate, is abundant in coffee, fruits, and vegetables, and is the primary source of CA in the human diet [3]. For regular consumers of coffee in Western countries, the major dietary phenolics are chlorogenic acids [4].

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The solubility of HCs is increased through thioester conjugation to various compounds. Although there is growing evidence for their beneficial health effects, the fruits and vegetables that contain high levels of HC conjugates are of limited availability in some parts of the world. Thus, there is growing interest in producing HC conjugates as nutraceutical supplements. The primary strategy for obtaining diverse phytochemicals is by extracting them from plants. However, this approach faces several obstacles such as a limited availability of plant materials and high costs of extraction and purification. Alternatively, they can be produced in microorganisms. Phytochemicals such as flavonoids and stilbenes have been produced using microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* [5-7].

Biosynthesis of HC conjugates is mediated by hydroxycinnamoyl transferases (HCTs), which use the coenzyme A thioester of HCs as a donor and various compounds such as quinate, and shikimate as HC acceptors. The formation of coenzyme A thioester with HC is catalyzed by 4-coumaric acid: CoA ligase (4-CL) [8]. Thus, genes encoding 4-CL and HCT, are essential for producing HC conjugates from HC. HCTs from several plants have been characterized [8-12]. HCTs show specificity for not only the acyl group donor but also the acyl group acceptor. When the acyl group acceptors are shikimate and quinate, the resulting conjugates are *p*-coumaroyl-*O*-shikimate and chlorogenic acid, respectively [13,14].

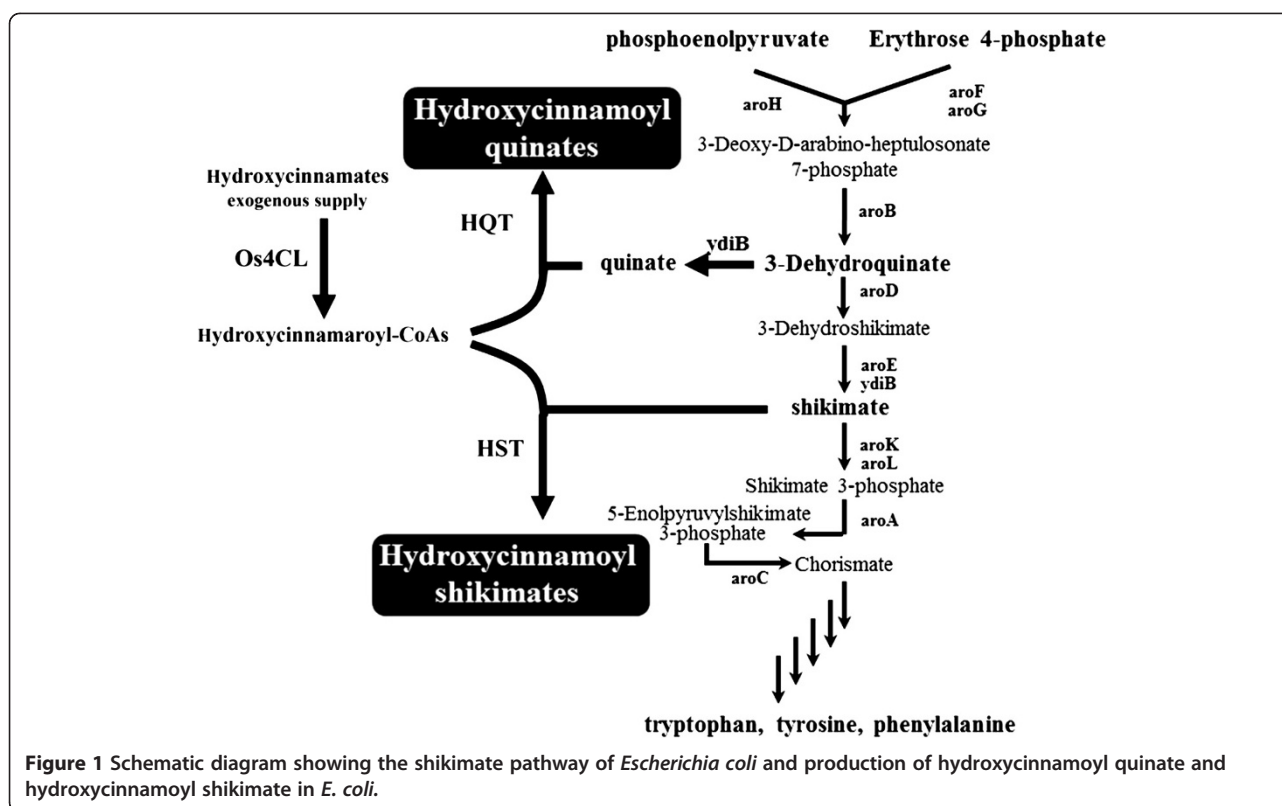
E. coli is a good system for producing plant secondary metabolites including phytochemicals produced through the phenylpropanoid pathway [15]. We attempted to produce HC conjugates using *E. coli*. Shikimate and 3-dehydroquininate, which are acyl group acceptors for hydroxycinnamate-CoA shikimate transferases (HST) and hydroxycinnamate-CoA quinate transferases (HQT), respectively, can be synthesized through the shikimate pathway of *E. coli* [16,17]. Thus, it should be possible to alter the shikimate pathway to increase the concentrations of shikimate and quinate. However, the acyl donor (PA or CA) is a unique product of plants [18]. In order to produce HCs in *E. coli*, coexpression of several genes in *E. coli* is necessary, which leads to metabolic load. Therefore addition of exogenous HCs may circumvent this problem. In this report, we introduced either HST and 4CL (for the production of CA-shikimate), or HQT and 4CL (for the production of chlorogenic acid) into *E. coli* mutants that accumulate either shikimate or 3-hydroquininate. When the engineered cells were fed exogenous HCs, a high yield of HC-shikimate and HC-quininate were obtained.

Results

Construction and selection of the optimum expression vector for the synthesis of HC-shikimate

HC-shikimate was synthesized from HC-CoA and shikimate using HST (Figure 1). Two genes, HST from *Nicotiana tabacum* (*NtHST*) and 4CL from *Oryza sativa* (*Os4CL*), were subcloned into an *E. coli* expression vector. *Os4CL* converts the HCs into the corresponding HC-CoAs. *Os4CL* has a better catalytic efficiency than other 4CLs [19]. *NtHST* uses HC-CoAs such as *p*-coumaroyl-CoA, caffeoyl-CoA, and feruloyl-CoA as acyl donors and shikimate as an acyl acceptor to produce HC-shikimate. *NtHST* was the first characterized HST and only a few number of HST has been biochemically characterized. *NtHST* also uses quinate as an acyl acceptor [9]. Both genes (*NtHST* and *Os4CL*) were subcloned into three different *E. coli* expression vectors, each of which has a different copy number in *E. coli*. Each construct (pA-*NtHST*-*Os4CL*, pC-*NtHST*-*Os4CL*, or pE-*NtHST*-*Os4CL*; see Table 1) was transformed into *E. coli* BL21(DE3) cells, and tested for its ability to produce HC-shikimate when incubated with exogenous PA. As a control, the empty vector was transformed into *E. coli*. HCs including PA can enter into *E. coli* via the 4-hydroxyphenylacetate permease (HpaX transporter) [20] and small phenolic compounds can be exported from *E. coli* into the culture medium. The transformant harboring pC-*NtHST*-*Os4CL* produced the highest concentration of HC-shikimate derivatives (29.1 mg/L), whereas the titers of HC-shikimate derivatives produced by the transformants harboring pA-*NtHST*-*Os4CL* or pE-*NtHST*-*Os4CL* were 24.4 and 27.4 mg/L, respectively. However, only PA was observed in the transformant harboring the empty vector. According to ANOVA test, a significant difference in PA-shikimates production at $P=0.01$ was shown between pC-*NtHST*-*Os4CL* and pA-*NtHST*-*Os4CL* or between pE-*NtHST*-*Os4CL* and pA-*NtHST*-*Os4CL*, but not between pC-*NtHST*-*Os4CL* and pE-*NtHST*-*Os4CL*. The construct present in the pCDF vector (pC-*NtHST*-*Os4CL*) was used in further studies because this construct has a lower copy number than pE-*NtHST*-*Os4CL*. Therefore, pC-*NtHST*-*Os4CL* is expected to impose a lower metabolic load on *E. coli*.

Although each vector exhibited varying biotransformation efficiencies, they all had the same reaction profiles. As shown in Figure 2, four new peaks appeared (P1 - P4). The molecular mass of P1 was 320.2 Da, indicating that one molecule of PA was attached to shikimate probably at the 5'-hydroxyl group of shikimate according to enzymatic studies [9,10]. In contrast, the molecular mass of the other three reaction products (P2 - P4) was 466.2 Da. Because the molecular mass of PA is 164 Da and that of shikimate is 174 Da, the three reaction products must be conjugates of two PA molecules and one



shikimate molecule. *p*-Coumaroyl shikimates are not commercially available and the structures of the reaction products was determined using nuclear magnetic resonance spectroscopy (NMR). By the help of the HMBC and HMQC experiments (Additional file 1: Figure S1, S2), 14 ¹³C peaks were determined, so that P1 was considered to be a conjugate of PA and shikimate. Even this structure contains 16 carbons, two set symmetric carbons of PA provide only two carbon peaks. Based on the HMBC spectrum (Additional file 1: Figure S1), the carboxyl group of PA was connected to the hydroxyl group of shikimate. Three isomers, the carboxyl group of PA connected to 3"-OH or 4"-OH or 5"-OH of shikimate, were possible. In the current NMR experiments, we could not determine the connected position. The name of the product P1 is (E)-3,4-dihydroxy-5-(3-(4-hydroxyphenyl)acryloyloxy)cyclohex-1-enecarboxylic acid where the bold number 5 could be switched with 3 or 4.

Based on the molecular masses of P2, P3, and P4 (Figure 2C), they contained two PAs and one shikimate. Two PAs can be connected to 3"-OH or 4"-OH or 5"-OH of SA, so that there can be three isomers, i.e. 3"-OH/4"-OH, 3"-OH/5"-OH, or 4"-OH/5"-OH. We could not determine which produce was which isomer. By comparison to a previous report [21], P3 and P4 are likely to be 3,5-di-*p*-coumaroyl shikimate and 4,5-di-*p*-coumaroyl shikimate, respectively. The remaining peak, P2 is therefore likely to be 3,4-di-*p*-coumaroyl shikimate.

Using *E. coli* harboring pC-NtHST-Os4CL, we determined the best acyl donor among CA, PA, and FA. In a previous study, recombinant NtHST protein most efficiently used caffeoyl-CoA as an acyl donor [9]. In this study, 1 mM of each HC was added to the same number of cells, and biotransformation was performed for 6 h. PA was the most effective acyl donor, producing 23.9 mg/L of *p*-coumaroyl shikimate. The amounts of feruloyl shikimate and caffeoyl shikimate produced were 3.8 mg/L, and 3.1 mg/L, respectively. Taken together, these results indicate that the highest amount of HC-shikimate was obtained with pC-NtHST-Os4CL as the construct and PA as the substrate.

Engineering *E. coli* to increase production of PA-shikimate conjugates

Because PA was added to the *E. coli* culture as an acyl donor, it was not a limiting factor for the production of PA-shikimate conjugates. However, *E. coli* uses endogenous shikimate as an acyl group acceptor. Thus, limiting shikimate availability would cause a bottleneck in the production of PA-shikimate conjugate. Previous studies showed that deletion of *aroK* and/or *aroL* in *E. coli* results in increased shikimate accumulation [22-24]. We created an *E. coli aroL* deletion mutant (BaroL), an *aroK* deletion mutant (BaroK) and an *aroK/aroL* double mutant (BaroKL) by gene replacement. pC-NtHST-Os4CL was transformed into each mutant and into wild type.

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

Plasmids or <i>E. coli</i> strains or Primers	Relevant properties or genetic marker	Source or reference
Plasmids		
pCYCDuet	P15A ori, Cm ^r	Novagen
pCDFDuet	CloDE13 ori, Str ^r	Novagen
pETDuet	f1 ori, Amp ^r	Novagen
pA-NtHST-Os4CL	pACYCDDuet carrying <i>NtHST</i> from <i>N. tobacco</i> and <i>4CL</i> from <i>O. sativa</i>	This study
pC-NtHST-Os4CL	pCDFDuet carrying <i>NtHST</i> from <i>N. tobacco</i> and <i>4CL</i> from <i>O. sativa</i>	This study
pE-NtHST-Os4CL	pETDuet carrying <i>NtHST</i> from <i>N. tobacco</i> and <i>4CL</i> from <i>O. sativa</i>	This study
pC-NtHQT-Os4CL	pCDFDuet carrying <i>NtHQT</i> from <i>N. tobacco</i> and <i>4CL</i> from <i>O. sativa</i>	This study
pA-EcydIB	pACYC carrying <i>ydiB</i> from <i>E. coli</i>	This study
Strains		
BL21 (DE3)	F ⁻ <i>ompT hsdS_B(r_B- m_B-) gal dcm lon</i> (DE3)	Novagen
B-100	BaroD carrying pCDF-Duet	This study
B-101	BaroD carrying pC-NtHQT-Os4CL	This study
B-102	BaroD carrying pC-NtHQT-Os4CL and pA-EcydIB	This study
BaroD	BL21(DE3) Δ <i>aroD::FRT-kan^R-FRT</i>	This study
BaroK	BL21(DE3) Δ <i>aroK::FRT-kan^R-FRT</i>	This study
BaroL	BL21(DE3) Δ <i>aroL::FRT-kan^R-FRT</i>	This study
BaroKL	BL21(DE3) Δ <i>aroK::FRT \Delta</i> <i>aroL::FRT-kan^R-FRT</i>	This study
Primers		
NtHST-F ¹	AACATATGAAGATCGAAGTGAAAGAAT (NdeI site is underlined)	
NtHST-R ²	AACTCGAGTCAAAAAGTCATACAAGAACTTC (XhoI site is underlined)	
NtHQT-F	AAGATATCCATGGGAAGTGAAAAATGATGA (EcoRV site is underlined)	
NtHQT-R	AAGGTACCTCAAAATTCATACAAATACTT (KpnI site is underlined)	
ydiB-F	ATGAATTCGATGGATGTTACCGCAAATAC (<i>EcoRI</i> site is underlined)	
ydiB-R	CATGCGGCCGCTCAGGCACCGAACCCCATG (<i>NotI</i> site is underlined)	
aroK-F	gctgtctttttacgtaacttaccgggtgatttatcgccagagcggtaattaacctcactaaagggcg	
aroK-R	cccgcagacgagtgtatataaagccagaattagtgctttccagcatgtgtaatacagctactatagggctc	
aroL-F	atgacaccggtcttcgcccattgacactattgggaaaaccacgatgaattaacctcactaaagggcg	
aroL-R	gatgaaccttaagtatagggcgtcgaaaatacaaatgatcgtctgtgctaatcagctactatagggctc	
aroD-F	tggggtcgggtcctgacagcgtgaccgctgcagaaagggtaaaaatgaattaacctcactaaagggcg	
aroD-R	gggaggatattcccgccgaaatattgcttatgcctgatgtaaatagtaatacagctactatagggctc	
aroK-check-F	cgctgcctgcgttccatgat	
aroL-check-F	cgcgagctggagaagtggt	
aroK or aroL check-R	taatacagctactatagggctc	
aroD-check-F	ggcaaggggctgaacagttc	
aroD-check-R	gggaggatattcccgccgaa	

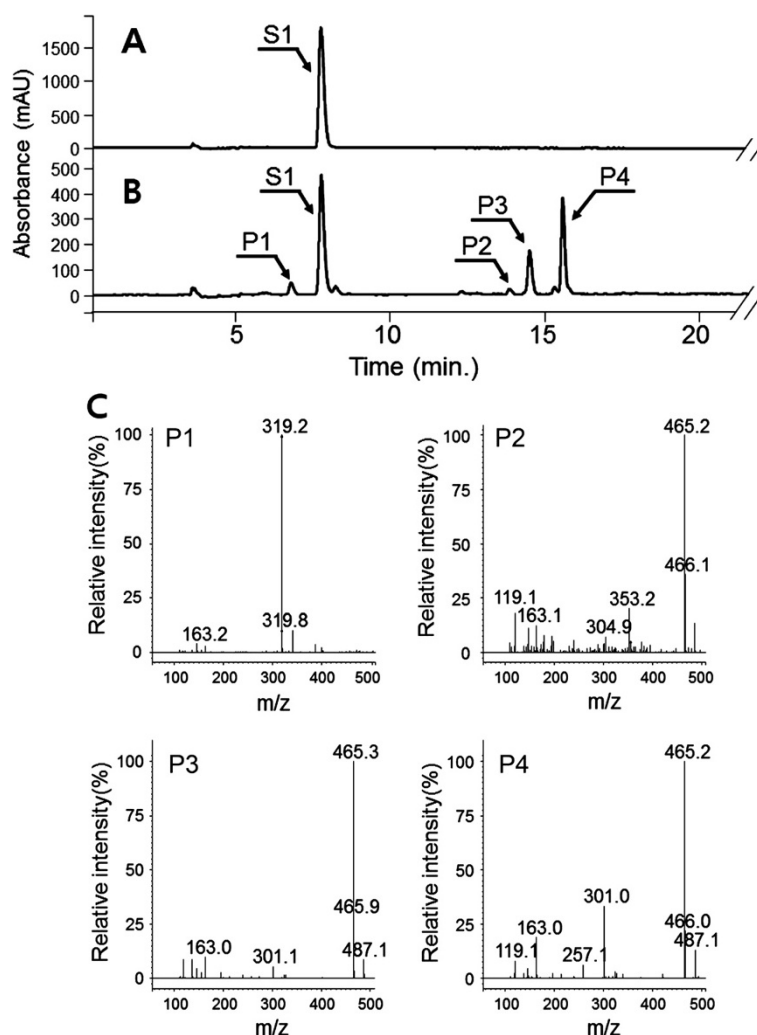
¹F means forward primer.

²R means reverse primer.

BaroL showed the highest productivity (approximately 235 mg/L), which is approximately 15-fold higher than BL21 (16 mg/L). BaroKL and BaroK produced 228 mg/L and 171 mg/L of PA-shikimates, respectively (Figure 3). According to ANOVA test, PA-shikimates production was significantly different at P=0.01 level among BL21,

BaroK, and BaroL. However, there was no difference between BaroL and BaroKL at this level of significance.

Next, production of PA-shikimate was optimized using BaroL harboring pC-NtHST-Os4CL. The optimum cell concentration was determined by varying the cell concentration from OD₆₀₀ 1 to 5 in the presence of 1 mM



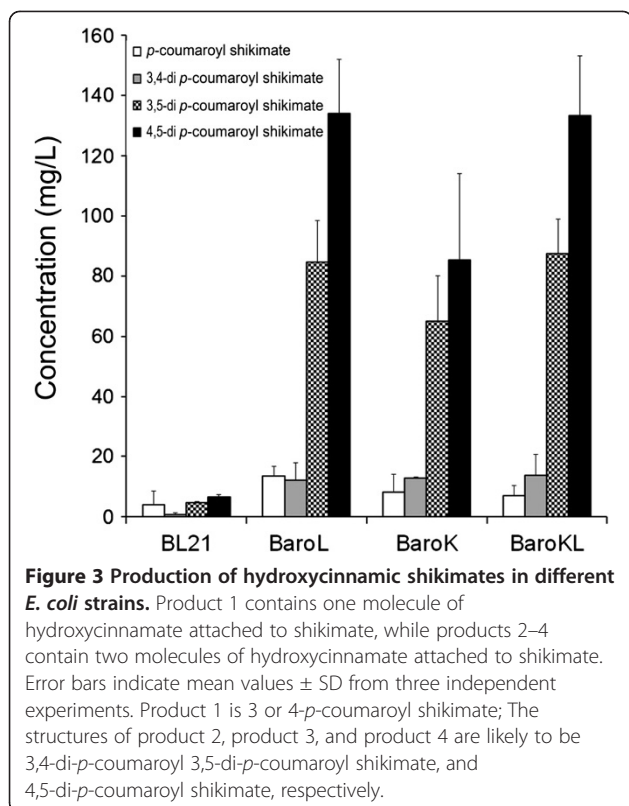
PA. Production of PA-shikimate peaked at an OD_{600} of 3, at which cell concentration the yield of PA-shikimate was approximately 235 mg/L. At $OD_{600} = 1$, or 2, the yield was approximately 94 mg/L, and 169 mg/L, respectively. Above $OD_{600} = 3$, the production of PA-shikimate decreased and was approximately 188 or 103.7 mg/L at $OD_{600} = 4$ or 5, respectively.

Using BaroL harboring pC-NtHST-Os4CL at a cell concentration of OD_{600} 3, we monitored the production of HC-shikimate over 12 h. The maximum amount of di-*p*-coumaroyl shikimates was produced at 8 h (134 mg/L 3,5-di-*p*-coumaroyl shikimate, 70 mg/L 4,5-di-*p*-coumaroyl shikimate, 8 mg/L of 3,4-di-*p*-coumaroyl shikimate, and 23 mg/L of 5-*p*-coumaroyl shikimate). Therefore, a total of 235 mg/L of PA-shikimates was produced. At 8 h, the amount of 4,5-di-*p*-coumaroyl

shikimate and 3,5-di-*p*-coumaroyl shikimate was reduced, but the amount of 5-*p*-coumaroyl shikimate reached a maximum (Figure 4). It seems that 5-*p*-coumaroyl shikimate was immediately converted to either 3,5-di-*p*-coumaroyl shikimate or 4,5-di-*p*-coumaroyl shikimate before 8 h.

Production of HC-quinate in *E. coli*

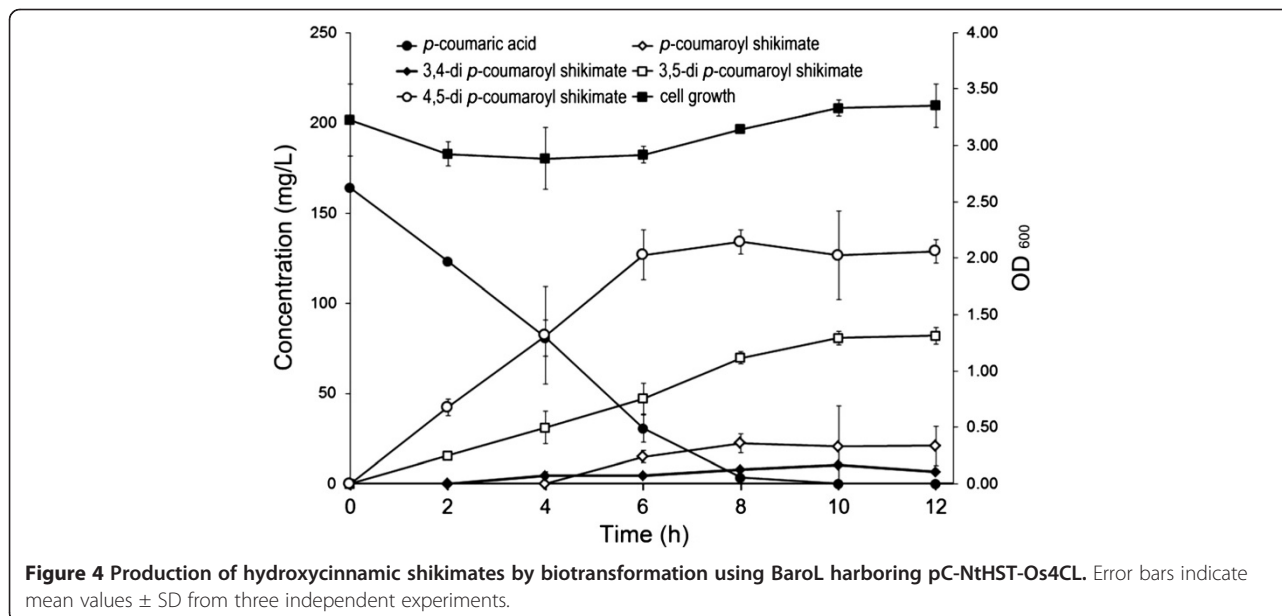
We used an acyltransferase that is specific for quinate to produce HC-quinate. Hydroxycinnamoyl-CoA quinate transferase from *N. tobacco* (NtHQT) was shown to be more specific for quinate than for shikimate [9,25]. Os4CL mediates formation of coenzyme A thioester with HC. To produce HC-quinate in *E. coli*, two genes, *NtHQT* and *Os4CL*, were subcloned into an *E. coli* expression vector. The resulting construct, pC-NtHQT-



Os4CL was transformed into BL21(DE3), and the transformant was used for biotransformation of CA. However, no reaction product was detectable. The amount of quinate or dehydroquinate in *E. coli* may not be high enough to drive the production of chlorogenic acid. Therefore, we created strain BaroD, which should accumulate higher levels of dehydroquinate and/or quinate,

in order to overcome this potential limitation. The strain BaroD was used as a host for pC-NtHQT-Os4CL and the resulting transformant was called B-101 (Table 1). As control, empty pCDFDuet was transformed into the strain BaroD and this transformant was called B-100 (Table 1). Each transformant was used for biotransformation of CA. The culture medium of B100 became a brown color after 8 h. Analysis of the reaction mixture using HPLC after 8 h incubation showed a trace level of caffeic acid. In addition, no detectable new product was observed. The decrease of caffeic acid during biotransformation is associated with the formation of *O*-quinone and its polyaromatic derivatives [26]. However, analysis of the biotransformation mixture from strain B-101 showed a new peak (Figure 5C) and the color of the culture medium did not change. Once caffeic acid is converted into another compound, it cannot polymerize and therefore the color of the culture medium does not change. The molecular mass of the product was 352 Da, which was 2 Da less than the predicted molecular mass of the thioester of quinate and CA (Figure 5C and E). Based on the molecular weight of the reaction product and the known intermediates of the *E. coli* shikimate pathway, it is like that 3-dehydroquinate and not quinate was attached to CA. NtHQT utilized 3-dehydroquinate as an acceptor and caffeoyl-CoA as a donor to make caffeoyl-3-dehydroquinate. Expression of *ydiB* from *E. coli*, which converts dehydroquinate into quinate, also increased the production of HC ester yield because NtHQT was likely to have higher affinity for quinate than for dehydroquinate as acyl group acceptor.

The protein product of *ydiB* converts 3-dehydroquinate into quinate [27]. *ydiB* was overexpressed in *E. coli* strain



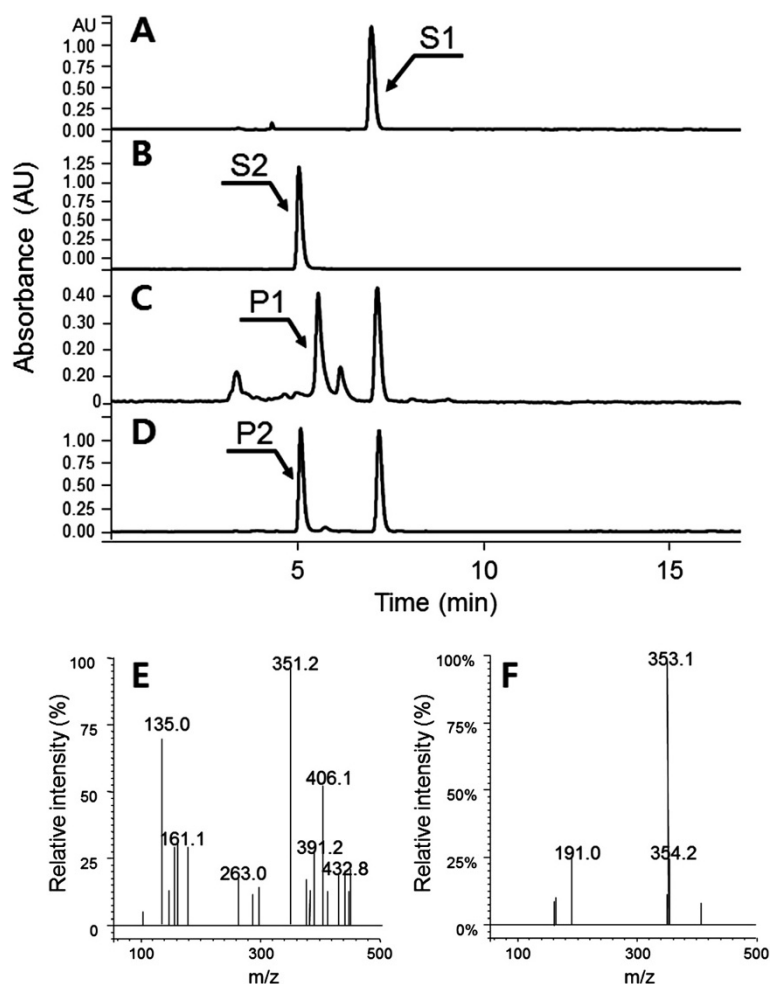


Figure 5 Production of chlorogenic acid in *E. coli*. **A**, caffeic acid (S1); **B**, chlorogenic acid (S2); **C**, reaction product of caffeic acid obtained from strain B-101; **D**, reaction product of caffeic acid obtained from strain B-102; **E**, MS/MS profile of P1; **F**, MS/MS profile of P2.

B-101 to make strain B-102. Biotransformation of CA by strain B-102 resulted in a new product with an identical HPLC retention time and molecular mass (354 Da) as chlorogenic acid (Figure 5D, F). The MS/MS spectrum of the reaction product matched authentic chlorogenic acid. This indicates that *ydiB* converts 3-dehydroquininate into quinate, which was then utilized for the production of chlorogenic acid.

PA and FA were also tested as acyl-group donors. CA was the best acyl-group donor followed by PA and FA. After 24 h, the amounts of caffeoyl-quinate, *p*-coumaroyl-quinate, and feruloyl-quinate were 450 mg/L, 323.7 mg/L, and 216 mg/L, respectively.

The production of chlorogenic acid was monitored further in strain B-102. After induction, 1.1, 1.3, 1.5, 1.7, or 2.0 mM CA was added to the culture. After 24 h, almost all of the CA was converted into chlorogenic acid in the reaction mixtures supplied with 1.1, 1.3, or 1.5 mM of CA. However, at a concentration of 1.7 or 2.0 mM, some CA

remained in the mixture, and even after a longer incubation time, the remaining caffeic acid was not converted into chlorogenic acid. However, the color of the mixture became dark brown due to the oxidation/polymerization of CA. Therefore, we monitored chlorogenic acid production at a CA concentration of 1.5 mM. As shown in Figure 6, production of chlorogenic acid continued to increase until 24 h, while the CA content decreased. Approximately 450 mg/L of chlorogenic acid was produced at 24 h.

Discussion

Anthocyanins, flavonoids, stilbene, and other compounds of plant origins have been biologically synthesized using engineered *E. coli* strains [7,15]. Most of these efforts involved introduction of new genes with high activity into *E. coli*. In addition, cofactor supplementation and reducing equivalents have been enhanced to produce natural compounds using engineered *E. coli* [6,28]. The goal of this study was to produce PA-

shikimate and chlorogenic acid in *E. coli*. We also engineered the shikimate pathway of *E. coli* to accumulate the acyl group acceptors, shikimate and quinate, which led to the increased production of PA-shikimates and chlorogenic acid.

During biotransformation, two PA molecules are attached to shikimate, whereas only one molecule of CA is attached to quinate. In plants, one molecule of CA is bound to one molecule of shikimate. However, diverse HC-quinatone conjugates, including those in which two or three identical HCs are bound to quinate, and two or three different HCs are bound to quinate, were found in coffee (*Coffea robusta*) and sweet potato (*Ipomonea batatas*) [21,29]. In these plants, the mono-esters are present as major components whereas di- and tri-esters are present as minor components. NtHST and NtHQT may have different enzymatic properties from HCTs from coffee or sweet potato because di-*p*-coumaroyl shikimates and chlorogenic acid were synthesized using NtHST and NtHQT. One possible scenario is that the substrate binding pocket of NtHQT may be narrower than that of NtHST. Thus, HC-quinatone may not fit into the substrate binding pocket of NtHQT for the second round of acyl transfer reaction. Longer incubation of the recombinant NtHST enzyme with PA-CoA and shikimate resulted in the production of di-PA-shikimate (data not shown). The production of di-PA-shikimate was also observed with HST from *Populus euramericana* [13]. Using the properties of NtHST, diverse HC-shikimate conjugates could possibly be obtained and feeding of different concentrations of various HCs into the *E. coli* culture medium may result in production of various forms of HC-shikimate conjugates. Any biological activity including anti-oxidant activity of di-PA-shikimate compared to PA-shikimate has not been

tested. However, 8-*O*-4-diferulic acid showed better antioxidant activity than ferulic acid [30].

p-Coumaric acid can be synthesized from tyrosine by the action of tyrosine ammonia lyase (TAL). Therefore, synthesis of *p*-coumaroyl-shikimate or chlorogenic acid from glucose might be possible if a gene encoding TAL were expressed into *E. coli* harboring *NtHST* and *Os4CL* or into *E. coli* harboring *NtHQT*, *Os4CL*, *ydiB*, and *Sam5* (*Sam5* converts *p*-coumaric acid into caffeic acid [31]), respectively. However, it is expected that only a tiny amount of reaction product would be produced from glucose in a wild type strain because quinate and shikimate are not accumulated in the wild type *E. coli*. Use of strain BaroD or BaroL is not also feasible because tyrosine is not synthesized in these strains. Thus, supplementation with either caffeic acid or tyrosine is needed to synthesize *p*-coumaroyl-shikimate or chlorogenic acid in *E. coli*.

Wild type *E. coli* expressing NtHQT and Os4CL did not produce detectable amounts of chlorogenic acid or caffeoyl-dehydroquinatone after CA supplementation. In the wild type, dehydroquinatone may have been rapidly converted into another downstream compound of the shikimate pathway instead of accumulating. Moreover, the expression level of *ydiB*, which converts dehydroquinatone into quinate, was low. Only a small amount of quinate or dehydroquinatone will be expected to be present in the cell in the absence of overexpression of *ydiB* [32]. *ydiB* and *aroE* mediate the conversion reaction not only from dehydroquinatone to quinate but also from dehydroshikimate to shikimate. However, *aroE* prefers dehydroshikimate to dehydroquinatone [22], whereas *ydiB* has nearly the same catalytic efficiency for dehydroshikimate and dehydroquinatone [27,33]. Recent studies have shown that overexpression of *ydiB* does not increase shikimate,

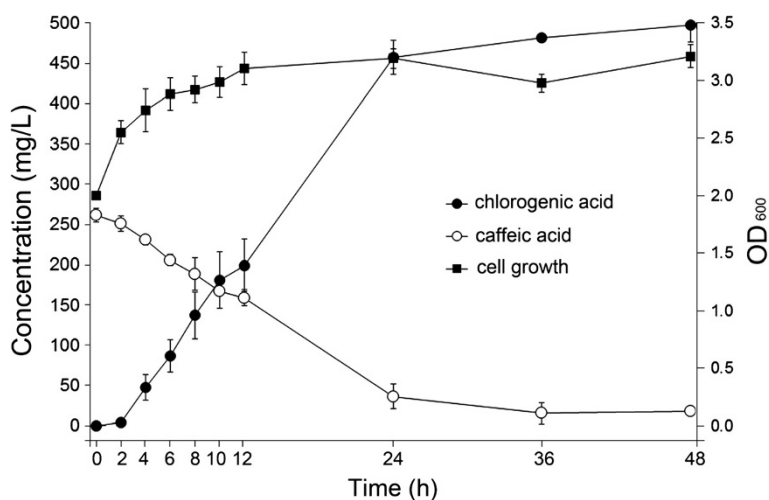


Figure 6 Production of chlorogenic acid using the *E. coli* strain B-106.

while overexpression of *aroE* increases conversion of dehydroshikimate to shikimate [32]. Therefore, we overexpressed *ydiB* to produce chlorogenic acid. In addition, strains BaroK, BaroL, and BaroKL expressing pC-NtHQT-Os4CL that were supplemented with CA produced chlorogenic acid only when *ydiB* was overexpressed (data not shown). This indicated that these mutants accumulate shikimate pathway intermediates such as dehydroquininate, dehydroshikimate and shikimate. These intermediates are converted into quinate by *ydiB*.

Conclusions

To produce the HC-conjugates in *E. coli*, several *E. coli* mutants including *aroD*, *aroK*, *aroL*, and *aroK/L* mutants were made and each mutant was tested using an optimized construct. For the production of HC-shikimates, an *E. coli aroL* mutant (B-*aroL*) was best and we produced 235 mg/L of HC-shikimates using B-*aroL* expressing *NtHST* and *Os4CL*, which is approximately 15-fold higher than wild type *E. coli* BL21 (16 mg/L). The wild type *E. coli* expressing *NtHQT* and *Os4CL* did not produce any detectable chlorogenic acid. By using *E. coli aroD* mutant (strain B-101), which accumulated 3-dehydroquininate, caffeoyl-3-dehydroquininate instead of chlorogenic acid, was synthesized. However, by expressing *ydiB* gene in strain B-101, which converted 3-dehydroquininate to quinate, chlorogenic acid production was dramatically increased up to 450 mg/L.

Materials and methods

Strains and reagents

The *E. coli* strains used in this study are listed in Table 1. *E. coli* BL21 (DE3) cells were used for recombinant protein production. *E. coli* DH5 α cells were used for plasmid cloning. All restriction enzymes and T4 DNA ligase were purchased from Takara (Shiga, Japan). Polymerase chain reaction (PCR) amplification was performed using Hotstart Taq DNA polymerase (Qiagen, Hilden Germany). Reverse transcription was performed using Omniscript reverse transcriptase (Qiagen). *E. coli* was cultured in Luria-Bertani (LB) or M9 medium (plus 2% glucose) containing 50 μ g/mL antibiotics, when necessary. *E. coli* expression vectors were purchased from Novagen (Madison, WI, USA).

Construction of *E. coli* expression vector

The genes for hydroxycinnamate-CoA shikimate transferase (*NtHST*) [9] and hydroxycinnamate-CoA quinate transferase (*NtHQT*) from tobacco (*N. tabacum*) [26] were cloned using reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from the leaves of one-month-old tobacco using Plant Total RNA Isolation Kit (Qiagen), and cDNA was synthesized

using Omniscript reverse transcriptase (Qiagen) and oligo dT as a primer. PCR was carried out using primers designed on the basis of the published sequences (GenBank accession AJ507825 for *NtHST* and AJ582651 for *NtHQT*). The primers were listed in Table 1. The *Os4CL* gene, which was cloned and characterized previously from rice [19], was subcloned into the *Bam*HI/*Not*I sites of pACYCDuet, pCDFDuet, and pETDuet vectors (EMD Chemicals, Gibbstown, NJ, USA), and then the resulting *NtHST* PCR product was subcloned into a second cloning site, the *Nde*I/*Xho*I site of each vector. The *NtHQT* PCR product was subcloned into the *Eco*RV/*Kpn*I site of pCDFDuet, which contains *Os4CL* at *Nde*I/*Xho*I site. The resulting constructs are listed in Table 1.

Deletion of the *aroL* and *aroK* genes in *E. coli* BL21 (DE3) was accomplished using the Quick and Easy Conditional Knockout Kit (Gene Bridges, Heidelberg, Germany). Briefly, the *aroL* gene or the *aroK* gene of *E. coli* BL21 (DE3) was replaced by the Δ *aroL* FRT-PGK-gb2-neo-FRT cassette or the Δ *aroK* FRT-PGK-gb2-neo-FRT cassette, respectively [34]. Deletion mutants were selected in Luria-Bertani (LB) medium containing 50 μ g/mL kanamycin. Deletion of *aroL* or *aroK* was confirmed using PCR. The strains deleted in *aroL* or *aroK* were named BaroL and BaroK, respectively (Table 1). Δ *aroL* and Δ *aroK* double mutant (strain BaroKL in Table 1) was constructed using strain BaroL. The kanamycin cassette was removed in Δ *aroL* using an FLP expression plasmid, which removes the kanamycin selection marker from the chromosome. Removal of the kanamycin cassette was confirmed by PCR. Using Δ *aroL* as a host, the *aroK* gene was replaced by the Δ *aroK* FRT-PGK-gb2-neo-FRT cassette. The primer sequences for the *aroD*, *aroK*, and *aroL* deletion are shown in Table 1.

Shikimate/quinate dehydrogenase gene, *ydiB* was cloned using *E. coli* BL21 (DE3) genomic DNA as a template. Primers were designed based on the published sequence (NC_000913.2) and the sequences of primers were listed in Table 1. The PCR product was digested with *Eco*RI/*Not*I and subcloned into the corresponding site of pCDF-Duet1. The resulting constructs were confirmed by sequencing.

Production of HC-shikimate conjugate in *E. coli*

Each construct was transformed into *E. coli* BL21 (DE3) strain or BaroL strain using electroporation with the BioRad MicroPulser Electroporation Apparatus (BioRad, Hercules, CA, USA). Overnight cultures of transformants were inoculated into LB medium containing 50 μ g/mL of antibiotic at 37°C and cultured until the OD₆₀₀ reached 0.8. Protein expression was induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), and growth was continued for another 20 h at 18°C. Cells were harvested and resuspended to obtain a cell concentration

corresponding to OD₆₀₀ of 3 in 2 mL of fresh M9 medium containing 2% glucose, 50 µg/mL of antibiotics, 1 mM IPTG, and 1 mM PA in a test tube (14 mm × 145 mm) and then cultured at 30°C for 8 h. The supernatant was extracted twice using an equal volume of ethyl acetate, and the upper aqueous phase was dried using a Speed Vac at 30°C, dissolved in 60 µL of dimethylsulfoxide (DMSO), and analyzed using high-performance liquid chromatography (HPLC). Because *p*-coumaroyl shikimate is not commercially available, we used *p*-coumaric acid to generate a standard curve for quantitative analysis of the reaction products. The UV spectra of *p*-coumaric acid are distinguishable from those of *p*-coumaroyl shikimate but they are very similar and the absorption of the thioesters is usually shifted to longer wavelength by a few nm. This is subtle enough that it should be perfectly acceptable to use the free acids as quantitation standards for the thioesters.

To determine the maximum conversion rate using the optimized vector, protein expression was induced as described above. The cell concentration was adjusted to an OD₆₀₀ of 3 in 25 mL of fresh M9 containing 2% glucose, 1 mM IPTG, and 50 µg/mL of antibiotics. The medium was supplemented with 1 mM PA. The reaction product (200 µL) was collected and extracted with ethyl acetate. The supernatant was dried and dissolved with 100 µL of dimethyl sulfoxide (DMSO). The sample was directly injected to HPLC for analysis. The mean and the standard error of the mean were calculated from triplicate experiments. Analysis of variance (ANOVA) was carried out using Tukey's method with a significance level of P=0.01 using 2010 Microsoft Office Excel.

Production of chlorogenic acid in *E. coli*

The construct for the production of chlorogenic acid was transformed into *E. coli* BL21(DE3) or BaroD cells. Induction of each construct was performed as described above. To determine the optimal gene construct, 200 µM PA was added to the growth medium.

To measure the production of chlorogenic acid in BaroD cells harboring pC-EcydIB and pC-NtHQT-Os4CL (Table 1), the cell concentration was adjusted to an OD₆₀₀ of 2.0, and CA was added to the medium to a final concentration of 1.5 mM. Production of chlorogenic acid was periodically monitored. The biotransformation was stopped by boiling for 5 min and the biotransformation product was centrifuged for 15 min at 13000 × g to remove the cell debris and other components prior to HPLC analysis. The quantification of the product was carried out using a standard curve generated with authentic chlorogenic acid (Sigma, MO, USA).

Analysis of the metabolites

The metabolites were analyzed using a Varian HPLC equipped with a photo diode array (PDA) detector and a

Varian C18 reversed-phase column (Varian, 4.60 × 250 mm, 3.5 µm particle size). The mobile phases consisted of 0.1% formic acid in water and acetonitrile. For chlorogenic acid, the program was: 20% acetonitrile at 0 min, 32% acetonitrile at 15 min, 90% acetonitrile at 17 min, 90% acetonitrile at 20 min, 20% acetonitrile at 21 min, and 20% acetonitrile at 26 min. To analyze hydroxycinnamoyl shikimate, the program was: 25% acetonitrile at 0 min, 40% acetonitrile at 10 min, 75% acetonitrile at 15 min, 90% acetonitrile at 22 min, 25% acetonitrile at 23 min, and 25% acetonitrile at 30 min. The flow rate was 1 mL/min, and the separation was monitored at 290 nm and 320 nm.

The molecular masses of the metabolites were determined using a Varian 500-MS ion trap spectrometer. Mass spectra were acquired simultaneously using an electrospray ionization source in negative ionization mode at 600 V. NMR spectrometry was done as described before [35].

Additional file

Additional file 1: Figure S1. The HMBC spectrum of the product P1.
Figure S2. The HMQC spectrum of the product P1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JHA initiated and coordinated the project. BGK, JWD, HM, and JHA performed experiments, analyzed data and wrote the paper. All authors approved the final manuscript.

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