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Caffeic acid production from glucose using metabolically engineered *Escherichia coli*

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Abstract

Caffeic acid (3,4-dihydroxycinnamic acid) is a precursor for high-valued compounds with anticancer, antiviral activities, and anti-inflammatory making it an important substance in the food additive, cosmetics, and pharmaceutical industries. Here, we developed an engineered *Escherichia coli* strain capable of directly producing high levels of caffeic acid from glucose. Tyrosine ammonia-lyase from *Rhodotorula glutinis* (RgTAL) and *p*-coumaric acid 3-hydroxylase from *Saccharothrix espanaensis* (SeC3H) were expressed. Next, feedback-resistant chorismate mutase/prephenate dehydrogenase, was introduced to promote L-tyrosine synthesis. This engineered strain CA3 produced 1.58 g/L of caffeic acid from glucose without tyrosine supplemented to the medium. Furthermore, to reduce *p*-coumaric acid accumulation, 4-hydroxyphenylacetate 3-hydroxylase from *Pseudomonas aeruginosa* (PaHpaBC) was introduced. Finally, an engineered strain CA8 directly produced 6.17 g/L of caffeic acid from glucose using a jar fermenter. The *E. coli* developed in this study would be helpful as a chassis strain to produce value-added caffeic acid-derivatives.

Keywords: caffeic acid, *E. coli*, metabolic engineering, shikimate pathway, *p*-coumaric acid

1. Introduction

Caffeic acid (3,4-dihydroxycinnamic acid) is a type of phenylpropanoid, a naturally occurring organic compound of plant origin. Caffeic acid has pharmacological activities, such as antioxidant [1], antiviral [2], anticancer [3], and antidepressant [4] effects, making it an important substance in the food additive, cosmetics, and pharmaceutical industries. The most common method of its production is extraction, mainly from plants, but the yield is low due to low accumulation in plant cells [5-7], also separation and purification are complicated and inefficient [8]. Therefore, as demand increases, the establishment of more efficient methods is desired, and biosynthesis via metabolically engineered microorganisms is attracting interest as a promising alternative [9].

Escherichia coli is widely used to produce many kinds of chemical compounds. Figure 1 shows the engineered metabolic pathway of caffeic acid biosynthesis in *E. coli*. Caffeic acid can be synthesized from tyrosine via a two-step reaction by tyrosine ammonia-lyase and *p*-coumaric acid 3-hydroxylase (C3H). In plants, cinnamic acid is formed from L-phenylalanine by phenylalanine ammonia-lyase, followed by two cytochrome P450 monooxygenases, cinnamic acid 4-hydroxylase (C4H) and *p*-coumaric acid 3-hydroxylase (C3H), which in turn produce *p*-coumaric acid, before it is converted to caffeic acid [10]. However, cytochrome P450 monooxygenases are difficult to express in several microbial systems [11-14]. In microbial systems, sam5-encoded SeC3H (from *Saccharothrix espanaensis*) can be used, allowing caffeic acid production in *Escherichia coli* [15,16]. Other effective hydroxylase complexes 4HPA3H for caffeic acid synthesis are EcHpaBC (from *Escherichia coli*) [11, 17], TtHpaBC (from *Thermus thermophilus HB8*) [18], RpHpaBC (from *Rhodopseudomonas palustris*) and PaHpaBC (from *Pseudomonas aeruginosa*) [19,20]. They have been reported, to show a high affinity for *p*-coumaric acid.

Previously, studies have been conducted to synthesize caffeic acid using the precursors *p*-coumaric acid and L-tyrosine as substrates [16,21]. However, caffeic acid synthesis relying on direct precursor

supply is undesirable due to high production costs. Therefore, a production process that allows utilization of a simple carbon source such as glucose has been developed [12, 16]. The reported caffeic acid titer using glucose as a carbon source is 1.03 g/L in *Escherichia coli* [22], 7.92 g/L in a 5-L fermenter using *Escherichia coli* [23] and 5.5 g/L in *Saccharomyces cerevisiae* [24, 25].

This study constructed caffeic acid-producing *E. coli* strain using metabolically engineering techniques. We previously developed a CFT1 strain [26,28], useful for aromatic compound biosynthesis via the shikimate (SHK) pathway. Therefore, it was selected as the parent strain. To construct the caffeic acid synthesis pathway, RgTAL (from *Rhodotorula glutinis*) and SeC3H (from *Saccharothrix espanaensis*) were expressed using plasmids. Next, to construct a series of processes to produce caffeic acid from glucose, $tyrA^{fbr}$, encoding a feedback-resistant chorismate mutase/prephenate dehydrogenase, was introduced. Constructed tyrosine overproducing strain CA3 produced 1.58 ± 0.09 g/L of caffeic acid at a yield of 0.08 ± 0.00 g/g-glucose. Strain CA8, in which PaHpaC (from *Pseudomonas aeruginosa*) was additionally introduced for pathway enhancement, produced 3.61 ± 0.10 g/L of caffeic acid at a yield of 0.19 ± 0.01 g/g-glucose, which significantly increased productivity. Furthermore, strain CA8 was cultured in a bioreactor and produced 6.17 ± 0.19 g/L of caffeic acid in 90 hours of culture. The production volume and yield obtained in this study are the highest values reported in previous caffeic acid biosynthesis studies. They may even be applicable in studies involving ferulic acid and L-dopa, which are caffeic acid derivatives.

2. Materials and Methods

2.1 Strains and plasmids construction

The strains and plasmids used in this study are listed in Table 1. ATCC31882 and its derivative strains were used for caffeic acid production. NovaBlue competent cells (Novagen, Cambridge, MA, USA) were used for gene cloning. Polymerase chain reaction was performed using KOD FX Neo (TOYOBO, Osaka, Japan). Custom DNA oligonucleotide primers were synthesized by Invitrogen Custom DNA Oligos (Thermo Fisher Scientific, Tokyo, Japan) and are listed in Table S1. Codon-optimized foreign gene fragments RgTAL, SeC3H, PaHpaB, and PaHpaC were synthesized by

Invitrogen GeneArt Gene Synthesis service (Thermo Fisher Scientific).

Plasmids named pZE12-RS, pTrcHisB-RS, pHLA-RS, and pSAK-RS were prepared. Briefly, RgTAL gene fragment was amplified by PCR using the primer pair pZS-RgTAL_Fw and pZS-RgTAL_Rv with the RgTAL synthetic gene as a template. SeC3H gene fragment was amplified by PCR using the primer pair pZS-SeC3H_Fw and pZS-SeC3H_Rv with SeC3H synthetic gene as a template. The amplified fragments were cloned between the KpnI/HindIII site of pZE12-MCS or pSAK [27] and the resulting plasmids were designated pZE12-RS or pSAK-RS. RgTAL–SeC3H gene fragment was amplified by PCR using the primer pairs pTrc-RgTAL_Fw and pTrc-SeC3H_Rv, pHLA-RgTAL_Fw and pHLA-SeC3H_Rv with pZE12-RS as a template. The amplified fragment was cloned between the *Bgl*II/*Eco*RI site of pTrcHisB or between the *Bgl*II/*Xho*I site of pHLA [28] and the resulting plasmids were designated pTrcHisB-RS or pHLA-RS.

Plasmids named pSAK-BC, pSAK-B, and pSAK-C were prepared as follows. Briefly, PaHpaB gene fragment was amplified by PCR using the primer pair pS-PaHpaB_Fw and pS-PaHpaB_Rv with PaHpaB synthetic gene as a template. PaHpaC gene fragment was amplified by PCR using the primer pair pS-PaHpaC_Fw and pS-PaHpaC_Rv with PaHpaC synthetic gene as a template. The plasmids obtained by cloning the PaHpaBC, PaHpaB, and PaHpaC fragments between the KpnI/HindIII sites of pSAK, respectively, were designated pSAK-BC, pSAK-B, and pSAK-C, respectively.

pSAK-aroG^{fbr} and pSAK-tktA were prepared as follows. Briefly, *aroG^{fbr}* was amplified by PCR using the primer pair pS-aroG^{fbr}_Fw and pS-aroG^{fbr}_Rv with *aroG^{fbr}* synthetic gene as a template. *tktA* was amplified by PCR using the primer pair pS-tktA_Fw and pS-tktA_Rv with ATCC31882 genomic DNA as a template. The plasmids obtained by cloning the *aroG^{fbr}* or *tktA* fragments between the KpnI/HindIII sites of pSAK were designated pSAK-aroG^{fbr} or pSAK-tktA.

A plasmid for *pheA* gene disruption named pTΔpheA was constructed as follows. Briefly, pTargetF [25] served as a template and was amplified by PCR of linearized fragments using the primer pairs del_pheA.N20_Fw and del_pheA.N20_Rv. The amplified fragments were self-ligated, and the resulting plasmid was designated pTFΔpheA. The upstream and downstream DNA sequences of *pheA* were amplified using the primer pairs del_pheA.UP_Fw and del_pheA.UP_Rv, del_pheA.DOWN_Fw and del_pheA.DOWN_Rv with ATCC31882 genome DNA as template. The amplified fragments were

cloned into the EcoRI/HindIII site of pTFΔpheA, and the resulting plasmid was designated pTΔpheA. pTΔpheA::$P_{A1lacO1}$-PaHpaB was constructed as follows. Briefly, pSAK-B served as a template and was amplified by PCR using the primer pairs del_pheA.pSAKinsert_Fw and del_pheA.pSAKinsert_Rv. The resulting fragment was cloned between the Spe I sites of pTΔpheA and the resulting plasmid was designated pTΔpheA::$P_{A1lacO1}$-PaHpaB. pTΔpheA::$P_{A1lacO1}$-PaHpaC, pTΔpheA::$P_{A1lacO1}$-aroG^{fbr}, and pTΔ pheA::$P_{A1lacO1}$-tktA were constructed by the same procedure. Disruption of *trpE*, *pheA*, *pykA* and *pykF* and transduction of *tyrA^{fbr}*, PaHpaB, PaHpaC, *aroG^{fbr}* and *tktA* were performed using the CRISPR-CAS9-plasmid system [29] with the following pTΔtrpE, pTΔpheA, pTΔpykA, pTΔpykF, pTΔtrpE::P_{trc}-tyrA^{fbr}, pTΔtrpE::$P_{A1lacO1}$-tyrA^{fbr} [26, 30], pTΔpheA::$P_{A1lacO1}$-PaHpaB, pTΔpheA::$P_{A1lacO1}$-PaHpaC, pTΔpheA::$P_{A1lacO1}$-aroG^{fbr}, and pTΔ pheA::$P_{A1lacO1}$-tktA. The deletion strains were constructed according to previous report [31].

2.2 Medium

LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) was used for pre-culture and culture during strain genetic manipulation. M9Y medium (20 g/L glucose, 5 g/L yeast extract, 40 mg/L L-tryptophan, 40 mg/L L-phenylalanine, 0.5 g/L NaCl, 1 g/L NH_4Cl, 3 g/L of KH_2PO_4, 6.7 g/L of Na_2HPO_4, 2.78 mg/L $FeSO_4 \cdot 7H_2O$, 14.7 mg/L $CaCl_2 \cdot 2H_2O$, 246 mg/L $MgSO_4 \cdot 7H_2O$, 10 mg/L of thiamine hydrochloride, 0.1 mM IPTG, 100 mg/L of ampicillin (and 30 mg/L of chloramphenicol)) was used for the caffeic acid production. The medium for bioreactor culture contained 50 g/L glucose. If needed, 0.5 g/L of L-tyrosine was added in the culture medium.

2.3 Culture conditions

E. coli colonies from LB plates were inoculated into test tubes containing 5 mL of LB medium and preincubated at 37°C for 24 h with shaking at 220 rpm. For test tube scale incubation, test tubes containing 5 mL of M9Y medium were inoculated with the pre-culture solution to an OD_{600} of 0.10, incubated at 37°C at 220 rpm, and 300 μL of the solution was collected at 24 and 48 h, which was then centrifuged at 10000 rpm for 20 min, and the supernatant was analyzed. For bioreactor cultures, 5 mL

of the pre-cultures in LB medium were transferred into flasks with 20mL of fresh medium and incubated at 37°C for an additional 8 h with shaking at 220 rpm. The pre-culture solution was then inoculated into a bioreactor containing 500 mL of M9Y medium to an OD_{600} of 0.10, incubated at 37°C. The pH was maintained at 7.00 by the automated addition of 28% (v/v) NH_3. Foam formation was suppressed using KM-70 (Shin-Etsu Chemical, Co., Ltd., Tokyo, Japan). Dissolved oxygen was maintained at >20% by controlling agitation. The solution (300 μL) was collected every predetermined time up to 96 h.

2.4 Analytical methods

Cell proliferation was evaluated using a UVmini-1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at an optical density of OD_{600}. For glucose analysis, a Prominence HPLC System (Shimadzu Corporation) equipped with a Shodex SUGAR KS-801 column (particle size 6 μm, L × I.D. 300 × 8.0 mm, Shodex) was used. The HPLC profile was monitored using a refractive index detector. Caffeic acid and *p*-coumaric acid were analyzed using HPLC equipped with a MSII column (particle size 5 μm, L × I.D. 250 × 4.6 mm, Nacalai Tesque). A two-component solvent system was used. Solvent A was 0.2% phosphate buffer, and solvent B was methanol. The flow rate of the mobile phase was 1.0 mL min^{-1} and the column was maintained at 40°C. The gradient was started with an 80:20 mixture of A and B (0–15 min), shifted to a 50:50 mixture of A and B (15–20 min), and back to an 80:20 mixture of A and B (20–25 min). HPLC profiles were obtained using a 240 nm UV–VIS detector. L-Tyrosine was analyzed using HPLC equipped with a PBr column (particle size 5 μm, L × I.D. 250 × 4.6 mm, Nacalai Tesque). A two-component solvent system was used. Solvent A was 0.2% phosphate buffer, and solvent B was methanol. The flow rate of the mobile phase was 1.0 mL min^{-1} and the column was maintained at 40°C. The gradient was started with 90:10 mixture of A and B (0–6 min), shifted to a 50:50 mixture of A and B (6–8 min), and back to 90:10 mixture of A and B (11–13 min). HPLC profiles were obtained using a 240 nm (caffeic acid and *p*-coumaric acid) or 220 nm (L-tyrosine) UV–VIS detector. The retention time of caffeic acid, *p*-coumaric acid and L-tyrosine were 4.0 min, 5.0 min and 10.0 min, respectively.

3. Results and Discussion

3.1 Construction of a caffeic acid production pathway in *E coli*

The previously developed *E. coli* CFT1 strain derived from ATCC 31882 was selected as the parent strain for this study. The endogenous phosphotransferase system (PTS) of CFT1 was replaced with a galactose permease/glucokinase system (GalP/Glk system). This modification increased intracellular phosphoenolpyruvate (PEP) utilization, an important factor in aromatic compound biosynthesis. First, we generated the CA0 strain based on CFT1 by disrupting *pheA*, which encodes prephenate dehydratase. PheA catalyzes competitive reactions in caffeic acid synthesis such as phenylalanine production. Subsequently, RgTAL and SeC3H were expressed in plasmids to construct a caffeic acid production pathway. Next, RgTAL and SeC3H were cloned into pZE12, pTrcHisB, pHLA, and pSAK vectors to create plasmids pZE12-RS, pTrcHisB-RS, pHLA-RS, and pSAK-RS. pZE12, pTrcHisB and pHLA are high-copy plasmids carrying LlacO1 promoter, Trc promoter and HCE promoter. pSAK is a low-copy plasmid carrying A1lacO1 promoter. These plasmids were respectively transformed into CA0. The caffeic acid conversion capacity of each strain was evaluated by adding 0.5 g/L of L-tyrosine to the medium. The results of the cultivation are shown in Figure 2. A strain CA0 harboring pZE12-RS showed the highest caffeic acid conversion capacity (0.47 ± 0.02 g/L) from L-tyrosine. The amount of p-coumaric acid was less than 0.1 g/L in all strains (data not shown). It indicates LlacO1 promoter was suitable for expression of RgTAL and SeC3H. Thus, pZE12-RS was selected as the plasmid for RgTAL and SeC3H expression. All of the following experiments were performed without the addition of tyrosine.

3.2 Production from glucose by $tyrA^{fbr}$ overexpression

Caffeic acid production using the strain CA0 harboring pZE12-RS was only observed when L-tyrosine was added. This strain did not produce caffeic acid from glucose (data not shown). This suggests that the amount of intracellular L-tyrosine must be increased to produce caffeic acid from glucose. Therefore, to promote L-tyrosine synthesis, $tyrA^{fbr}$, encoding feedback-resistant chorismate mutase/prephenate dehydrogenase, was introduced into the CA0 strain in three ways. A low-copy

plasmid for *tyrA*^{fbr} expression under the Trc promoter (pSAK-Ptrc-tyrA^{fbr}) control, was constructed. A CA0 strain harboring pZE12-RS and pSAK-Ptrc-tyrA^{fbr} was named CA1. A strain in which *tyrA*^{fbr} was integrated into the *trpE* locus of the genome under the Trc promoter named CA2 and a strain in which *tyrA*^{fbr} was introduced into the same locus under the A1lacO1 promoter was named CA3. Caffeic acid production after 48 h using glucose as a sole carbon source is shown in Figure 3. All strains introduced with *tyrA*^{fbr} successfully produced caffeic acid from glucose. Particularly, CA3 produced 1.58 ± 0.09 g/L, approximately 1.3-times more than strains CA1 and CA2. Therefore, CA3 was selected as the caffeic acid producing strain.

3.3 Enhancement of the caffeic acid production pathway by PaHpaB and PaHpaC overexpression

After CA3 cultivation, the accumulation of *p*-coumaric acid (1.20 ± 0.10 g/L), caffeic acid precursor, was observed (Figure 3). This suggests that the reaction to convert *p*-coumaric acid to caffeic acid is the bottleneck of caffeic acid production. We attempted to enhance the reaction that converts *p*-coumaric acid to caffeic acid, however, additional expression of SeC3H could not improve caffeic acid production (data not shown). Other than SeC3H (from *Saccharothrix espanaensis*), EcHpaBC (from *Escherichia coli*) and TtHpaBC (from *Thermus thermophilus HB8*) have been reported to catalyze this reaction. EcHpaBC has high activity than TtHpaBC [18], and EcHpaBC-mediated caffeic acid producing pathway produced 12.1 mg/L of caffeic acid from glucose [11]. When yeast was used as a host, 569.0 mg/mL of caffeic acid was produced [24]. We focused on PaHpaBC (from *Pseudomonas aeruginosa*), which showed high oxidation activity [19], but they have not previously been used for the production of caffeic acid from glucose. Therefore, we introduced them into CA3. A CA3 strain expressing PaHpaBC and using plasmid pSAK-PaBC was named CA4. A strain CA5, with PaHpaB introduced into the genome of CA3 under the control of the A1lacO1 promoter, was also constructed. A strain CA6 is a CA3 strain carrying plasmid pSAK-PaB for PaHpaB expression only. Similarly, a CA7 strain was constructed by introducing PaHpaC in its genome under the control of the A1lacO1 promoter, and a CA8 strain carries a plasmid pSAK-PaC for HpaC expression. The results of the culture are shown in Figure 4. CA4, which has PaHpaBC introduced as a complex, showed a slight production decrease, while CA5-CA8, which had PaHpaB/PaHpaC independently introduced, showed

decreased *p*-coumaric acid accumulation and increased caffeic acid production. Particularly, CA8 produced 3.61 ± 0.09 g/L of caffeic acid, approximately 2.3-times more than CA3. CA7 produced 3.11 g/L of caffeic acid, which is higher than CA6 (1.88 g/L) and CA5 (1.91 g/L). The accumulation of *p*-coumaric acid were less than 0.6 g/L among all strains (Figure 4) and no L-tyrosine was detected in the medium (data not shown). Strains CA7 and CA8 overexpressed PaHpaC and CA5 and CA6 overexpressed PaHpaB. SeC3H is a single-component $FADH_2$-utilizing hydroxylases and FAD is regenerated such as a flavin reductase in *E. coli* [32]. PaHpaBC are a two-component flavin-dependent monooxygenase consists of reductase (PaHpaC) and oxygenase (PaHpaB) [33]. Generally, optimization of $FADH_2$ transfer within or between flavin-dependent enzymes is important to decrease the extravagant consumption of reducing agents NADH. From the Figure 4, the enhancement of reduced flavin supply (i.e. PaHpaC overexpression) improved caffeic acid production.

3.4 Enhancement of upstream pathways related to E4P/PEP

After caffeic acid biosynthesis pathway was established in *E. coli* through metabolic engineering of the downstream pathway after chorismic acid, we focused on enhancing the upstream pathway to achieve even higher caffeic acid production. The methods of shikimate pathway enhancement have been reported previously [27]. The first step of the SHK pathway is the condensation of erythrose 4-phosphate (E4P) and PEP to produce 3-deoxy-d-arabinoheptulosonic acid 7-phosphate (DAHP) by the action of DAHP synthase. DAHP synthase of *E. coli* is composed of three isozymes, AroG, AroF, and AroH, whose activity is strictly regulated by allosteric regulation and transcriptional repression [35]. Therefore, to enhance DAHP synthesis response, $aroG^{fbr}$, encoding feedback-resistant AroG, was introduced into CA8 to create a CA9 strain. Furthermore, the CA8 strain shortened the intermediate substrates, E4P and/or PEP. Therefore, to promote E4P supply, the CA10 strain was generated by introducing *tktA*, which encodes a transketolase that catalyzes the E4P synthesis reaction, into CA8. In a report, PEP accumulation can be effectively controlled by the inactivation of pyruvate kinase I (*pykF*)/pyruvate kinase II (*pykA*), which catalyzes the reaction that converts PEP to pyruvate [35, 36]. Therefore, we generated a CA11 strain, which had *pykF*/*pykA* disrupted. Since the inactivation of pyruvate kinase I/pyruvate kinase II weakens the TCA cycle and affects bacterial growth, 2 g/L of

sodium pyruvate was added to the culture medium. The results of the cultivation are shown in Figure 5. Caffeic acid production was almost at similar levels among these strains, suggesting that E4P and PEP supply is not a limiting factor in caffeic acid production from glucose.

3.5 Caffeic acid production using a jar fermenter

The CA8 strain was cultured in a jar fermenter to demonstrate the potential for scaled-up production for industrialization. Modified M9Y medium with 50 g/L glucose was used. Figure 6 shows the culture profiles in the jar fermenter. Caffeic acid production using jar fermenter was 6.17 ± 0.19 g/L after 90 h cultivation, which is the highest titer from glucose reported to date. Almost all glucose was consumed during caffeic acid production. Less than 0.6 g/L of p-coumaric acid accumulated, but it was consumed during the cultivation. These results indicates that the caffeic acid-producing strains developed in this study are effective and have the potential to be scaled-up for industrialization.

4. Conclusion

We succeeded in constructing a caffeic acid-producing pathway from glucose in *E. coli* by metabolic engineering. We demonstrated that enhancement of PaHpaC expression effectively increased caffeic acid production. Using a jar fermenter, we succeeded in producing 6.17 g/L caffeic acid, the highest value obtained from glucose as a carbon source using *E. coli*. We believe that establishing the caffeic acid production process can serve as an effective platform technology for the synthesis of derivatives of other shikimate pathway intermediates.

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Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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CRediT authorship contribution statement

Kosuke Sakae: Conceptualization, Methodology, Investigation, Writing–Original Draft. Daisuke Nonaka: Investigation. Mayumi Kishida: Investigation. Yuuki Hirata: Investigation. Ryosuke Fujiwara: Methodology, Investigation, Writing–Review & Editing. Akihiko Kondo: Methodology. Shuhei Noda: Conceptualization, Methodology, Writing–Review & Editing. Tsutomu Tanaka: Conceptualization, Methodology, Investigation, Writing–Review & Editing.

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Figures

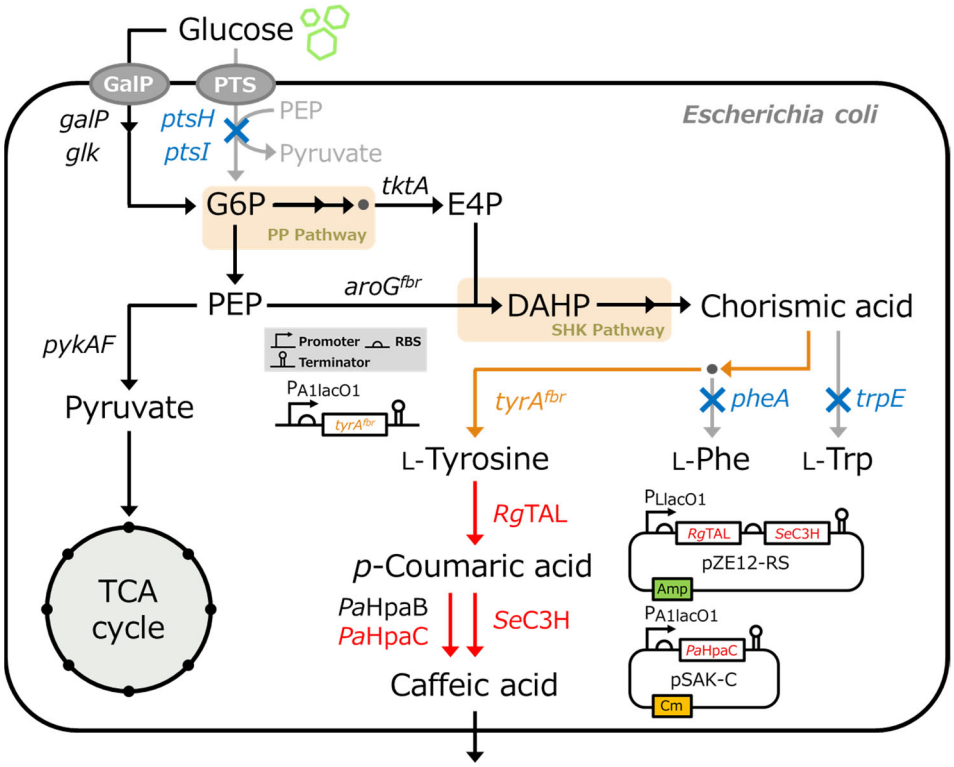
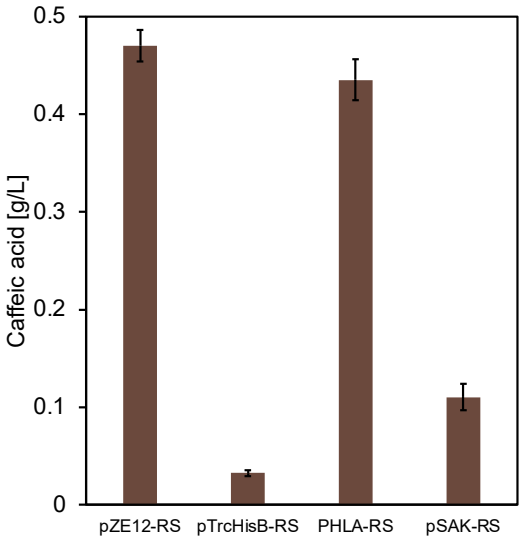
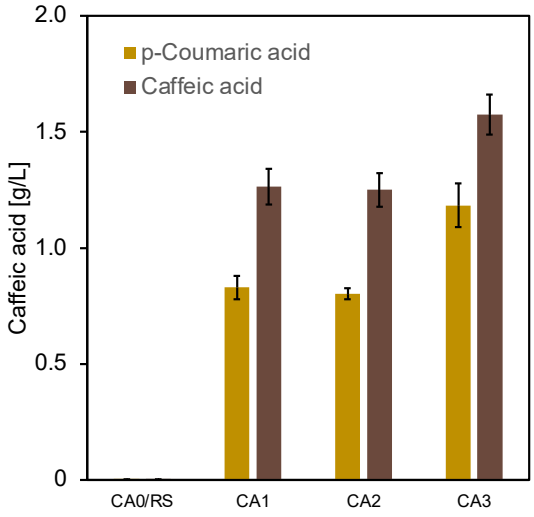


Figure 1. Metabolic engineering of caffeic acid producing *E. coli*. The blue X indicates disruption of the *ptsH*, *ptsI*, *trpE* and *pheA* genes. Red or orange shows genes involved in caffeic acid synthesis overexpressed by plasmid or genomic integration. G6P, glucose 6-phosphate; PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; DAHP, 3-deoxy-d-arabinoheptulosonic acid 7-phosphate; PP pathway, pentose–phosphate pathway; SHK pathway, shikimate pathway; RgTAL, tyrosine ammonia-lyase from *Rhodotorula glutinis*; SeC3H, *p*-coumaric acid 3-hydroxylase from *Saccharothrix espanaensis*; PaHpaBC, hydroxylase complexes 4HPA3H from *Pseudomonas aeruginosa*; $tyrA^{fbr}$, feedback-resistant chorismate mutase/prephenate dehydrogenase; *galP*, d-galactose transporter; *glk*, glucokinase; *ptsH*, phosphocarrier protein HPr; *ptsI*, phosphoenolpyruvate-protein phosphotransferase; *pykF*, pyruvate kinaseI; *pykA*, pyruvate kinaseII; *tktA*, transketolase; $aroG^{fbr}$, feedback-resistant 3-deoxy-7-phosphoheptulonate synthase; *trpE*, anthranilate synthase component I; *pheA*, chorismate mutase/prephenate dehydratase.



418

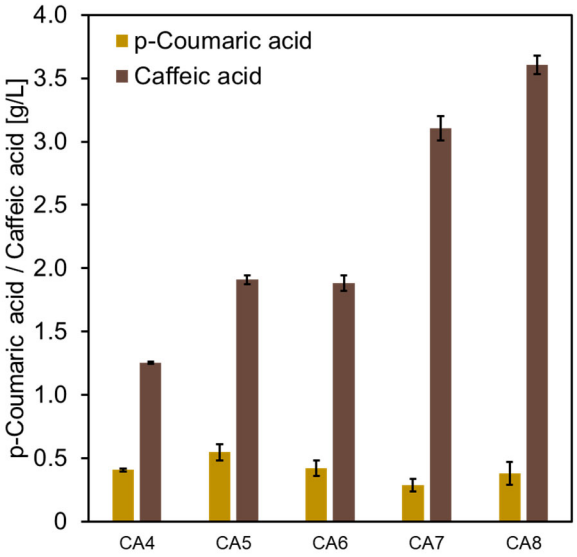
419 **Figure 2.** Caffeic acid production after 48 h of cultivation in M9Y medium containing 0.5 g/L L-
420 tyrosine and 20 g/L glucose using the strains harboring plasmid pZE12-RS, pTrcHisB-RS, pHLA-RS,
421 or pSAK-RS. The data shown are as the means and standard deviations of three independent
422 experiments.



423

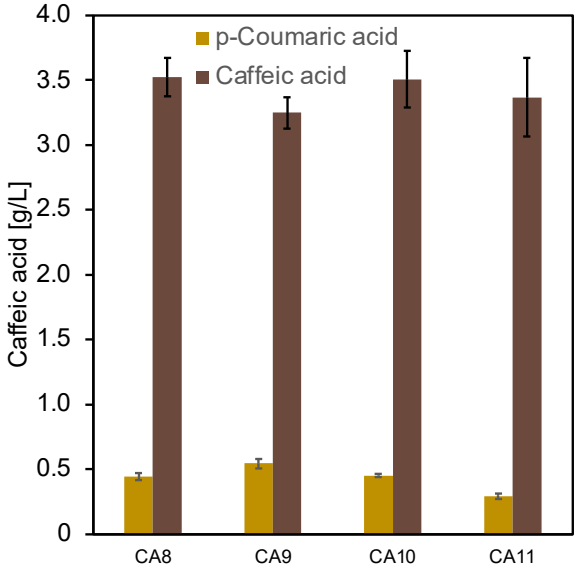
424 **Figure 3.** Caffeic acid production after 48 h of cultivation in M9Y medium containing 20 g/L glucose
425 using strains CA1, CA2, and CA3. The data shown are as the means and standard deviations of three
426 independent experiments

427



428

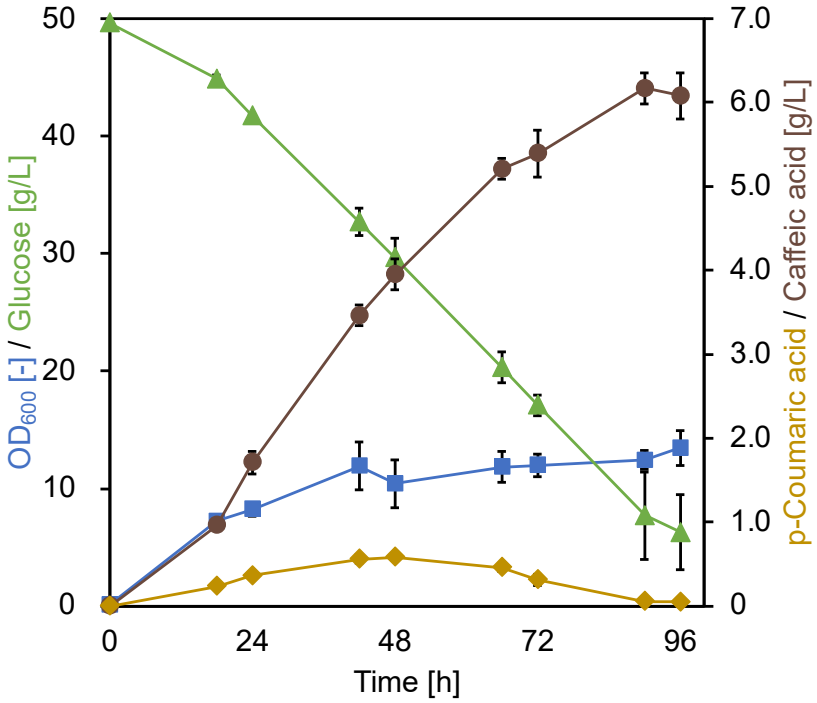
429 **Figure 4.** Caffeic acid production after 48 h of cultivation in M9Y medium containing 20 g/L glucose
430 using the strains CA4-CA8. The data shown are as the means and standard deviations of three
431 independent experiments.



432

433 **Figure 5.** Caffeic acid production after 48 h of cultivation in M9Y medium containing 20 g/L glucose
434 using the strains CA8-CA11. The data shown are as the means and standard deviations of three
435 independent experiments

436



437

438 **Figure 6.** CA8 culture profiles in a jar fermenter. Blue squares, green triangles, light-brown and dark-
439 brown symbols indicate cell growth, glucose concentration, *p*-coumaric acid concentration and caffeic
440 acid concentration, respectively. The data shown are as the means and standard deviations of three
441 independent experiments.

442

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

Strains and Plasmids		
Strains	**Genotype**	**Reference**
NovaBlue	*endA1 hsdR17*(*rK12-mK12*$^{+}$) *supE44 thi-1 gyrA96 relA1 lac recA1*/F [*proAB*$^{+}$ *laclqZΔM15 Tn10*(*TetR*)]	Novagen
ATCC31882	L-Phenylalanine-overproducing strain (aroF aroG tyrR pheA tyrA trpE)	ATCC
CFT1	ATCC31882Δ*ptsHI*::$P_{A1lacO1}$-*glk-galP*	Noda et al
CA0	CFT1Δ*pheA*	This study
CA0/RS	CFT1Δ*trpE*Δ*pheA*/pZE12-RS	This study
CA1	CFT1Δ*trpE*Δ*pheA*/pZE12-RS+pSAK-Ptrc-tyrA^{fbr}	This study
CA2	CFT1Δ*trpE*::P_{trc}-*tyrA*^{fbr}Δ*pheA*/pZE12-RS	This study
CA3	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*/pZE12-RS	This study
CA4	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*/pZE12-RS+pSAK-BC	This study
CA5	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*::$P_{A1lacO1}$-PaHpaB/pZE12-RS	This study
CA6	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*/pZE12-RS+pSAK-B	This study
CA7	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*::$P_{A1lacO1}$-PaHpaC/pZE12-RS	This study
CA8	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*/pZE12-RS+pSAK-C	This study
CA9	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*::$P_{A1lacO1}$-*aroG*^{fbr}/pZE12-RS+pSAK-C	This study
CA10	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*::$P_{A1lacO1}$-*tktA*/pZE12-RS+pSAK-C	This study
CA11	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*Δ*pykAF*/pZE12-RS+pSAK-C	This study
Plasmids	**Characteristic**	**Reference**

pZE12-MCS	P_{LlacO1}, *colE ori*, and Amp^r	Expressys
pTrcHisB	P_{trc}, *pBR322 ori*, and Amp^r	Life Technologies
pHLA	P_{HCE}, *colE1 ori*, and Amp^r	Tanaka et al., 2011
pSAK	$P_{A1lacO1}$, *SC101 ori*, and Cm^r	Noda et al., 2017
pZE12-RS	pZE12 containing RgTAL and SeC3H	This study
pTrcHisB-RS	pTrcHisB containing RgTAL and SeC3H	This study
pHLA-RS	pHLA containing RgTAL and SeC3H	This study
pSAK-RS	pSAK containing RgTAL and SeC3H	This study
pSAK-Ptrc-tyrA^{fbr}	pSAK-P_{trc} containing $tyrA^{fbr}$	Fujiwara et al., 2020
pSAK-BC	pSAK containing PaHpaBC	This study
pSAK-B	pSAK containing PaHpaB	This study
pSAK-C	pSAK containing PaHpaC	This study
pSAK-aroG^{fbr}	pSAK containing $aroG^{fbr}$	This study
pSAK-tktA	pSAK containing *tktA*	This study
pTargetF	Constitutive expression of sgRNA	Addgene
pCas	Constitutive expression of cas9 and inducible expression of λ RED and sgRNA	Addgene
pTΔtrpE	Constitutive expression of sgRNA with donor editing template DNA for *trpE* disruption	Fujiwara et al., 2020
pTΔtrpE::P_{trc}-tyrA^{fbr}	Constitutive expression of sgRNA with donor editing template DNA for P_{trc}-$tyrA^{fbr}$ into *trpE* gene loci	Fujiwara et al., 2020
pTΔtrpE::$P_{A1lacO1}$-tyrA^{fbr}	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-$tyrA^{fbr}$ into *trpE* gene loci	Fujiwara et al., 2020

pTΔpykA	Constitutive expression of sgRNA with donor editing template DNA for *pykA* disruption	Noda et al., 2016
pTΔpykF	Constitutive expression of sgRNA with donor editing template DNA for *pykF* disruption	Noda et al., 2016
pTΔpheA	Constitutive expression of sgRNA with donor editing template DNA for *pheA* disruption	This study
pTΔpheA::$P_{A1lacO1}$-PaHpaB	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-PaHpaB into *pheA* gene loci	This study
pTΔpheA::$P_{A1lacO1}$-PaHpaC	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-PaHpaC into *pheA* gene loci	This study
pTΔpheA::$P_{A1lacO1}$-aroG^{fbr}	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-*aroG*^{fbr} into *pheA* gene loci	This study
pTΔpheA::$P_{A1lacO1}$-tktA	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-*tktA* into *pheA* gene loci	This study

Graphical abstract