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Production of Genistein from Naringenin Using *Escherichia coli* Containing Isoflavone Synthase–Cytochrome P450 Reductase Fusion Protein

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Isoflavonoids are a class of phytoestrogens. Isoflavonone synthase (IFS) is responsible for the conversion of naringenin to genistein. IFS is a cytochrome P450 (CYP), and requires cytochrome P450 reductase (CPR) for its activity. Additionally, the majority of cytochrome P450s harbor a membrane binding domain, making them difficult to express in *Escherichia coli*. In order to resolve these issues, we constructed an in-frame fusion of the *IFS* from red clover (*RCIFS*) and *CPR* from rice (*RCPR*) after removing the membrane binding domain from RCIFS and RCPR. The resultant fusion gene, *RCIFS–RCPR*, was expressed in *E. coli*. The conversion of naringenin into genistein was confirmed using this *E. coli* transformant. Following the optimization of the medium and cell density for biotransformation, 60 μM of genistein could be generated from 80 μM of naringenin. This fusion protein approach may be applicable to the expression of other P450s in *E. coli*.

Keywords: Cytochrome P450, cytochrome P450 reductase, genistein, naringenin

Polyphenols, one group of plant secondary metabolites, include the (iso)flavonoids, cumarins, stilbenes, and lignins [2]. Among the polyphenols, more than 10,000 flavonoids have been identified thus far [18]. Flavonoids, like other phenylpropanoids, branch out from the primary metabolites (L-phenylalanine or L-tyrosine) *via* the phenylpropanoid pathway [6, 19]. The entry point of flavonoid biosynthesis is catalyzed by chalcone synthase, which mediates the condensation of three molecules of malonyl-CoA and one coumaroyl-CoA. The resultant chalcone naringenin is converted to naringenin either spontaneously or *via* the action of chalcone isomerase. Naringenin functions as a substrate for the biosynthesis of other flavonoids [19].

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Flavonoids are divided into seven major subgroups: chalcones, flavanones, (iso)flavones, flavonols, flavandiols, anthocyanins, and condensed tannins [19]. Among these subgroups, the isoflavone biosynthesis pathway is a branch of the flavone biosynthesis pathway, and is detected exclusively in leguminous plants. Isoflavones perform important roles in interactions with other organisms, such as in plant disease resistance or in nodulation [5, 19]. The impacts of isoflavones on humans (estrogenic, antioxidant, and anticancer activities) have been the focus of a great deal of attention in recent years [3, 4]. Isoflavone is synthesized *via* the migration reaction of an aryl ring of flavanone by IFS, cytochrome P450 enzyme CYP93C [1, 7, 11].

Despite the various biological activities of flavonoids, low productivity is a major obstacle to the use of flavonoids in medicinal food or drug products. Although chemical syntheses are available for some flavonoids, they typically involve toxic chemicals and extreme reaction conditions that render the entire process difficult to scale up. In order to circumvent these problems, microbial hosts such as *Escherichia coli* and yeast might be a useful tool for mass production. *E. coli* have been engineered to generate a variety of natural products from plants and other hard-to-culture organisms [8, 20, 21]. Using the *E. coli* system, curcuminoids, flavonols, anthocyanins, and stilbenes have been manufactured [9, 10]. The biosynthesis of these compounds involves several genes. CYPs including IFS, flavone synthase II, flavonoid 3' hydroxylase, and flavonoid 3',5' hydroxylase are involved in the hydroxylation, double-bond formation, or ring migration of flavonoids. To execute these reactions, CYP must provide electrons from NADPH with the help of CPR [13, 14, 17]. Both CYP and CPR harbor hydrophobic membrane binding domains at the N-terminus, which makes the expression of these proteins difficult, owing to low solubility. Cytochrome P450 can be expressed in *E. coli* if the hydrophobic membrane binding domain is eliminated, and the CPR gene can then be fused with the P450 gene to transfer an electron from NADPH [13, 17].

IFS, with the help of CPR, converts naringenin into 2,5,7,4'-tetrahydroxyisoflavanone, which almost spontaneously becomes genistein [1]. To generate genistein from naringenin, we constructed an in-frame fusion of IFS from red clover and CPR from rice after removing the membrane binding domains from RCIFS and RCPR. We describe the production of genistein using an *E. coli* variant containing this fusion gene.

Materials and Methods

DNA Manipulation

Restriction enzymes and T4 DNA ligase were purchased from Takara Biochemicals. Hot-start and Proof *Taq* polymerases were purchased from Qiagen (Qiagen, Gaithersburg, MD, U.S.A.). Flavonoids were purchased from Indofine Chemicals (Hillsborough, NJ, U.S.A.).

Construction of *RCIFS–RCPR* and *RCIFS–CCPR*

To remove the membrane binding domain and stop codon of *RCIFS*, we designed two primers on the basis of a previously published sequence (AY253284), with 5'-AAGAATTCATGACCGCTAAATC AAAAGCA-3' as forward and 5'-CTCCACCGGATCGGAACGTC GACCCAGAGGAAAGGAGTTT-3' as reverse primers. The forward primer starts at the 64th nucleotide, and the reverse primer of *RCIFS* harbors a Gly-Ser-Thr linker sequence (underlined above for reverse primer) and 15 *RCPR* nucleotides. *RCIFS* was amplified using two primers. Forty-nine amino acids from the N-terminal of RCPR, which is a membrane binding domain, were removed *via* a PCR method. Two primers, 5'-CTAAACTCCTTTCCTCTGGGTCGACGTTCCGATCCGG TGG-3' (forward) and 5'-AAGCGGCCGCTCACCATACGTCACGGAG GT-3' (reverse), were designed on the basis of the sequence listed under the GenBank accession number XP_474161. The forward primer contained a Gly-Ser-Thr linker sequence (underlined) and 15 nucleotides of *RCIFS*. *RCPR* was amplified with Hotstart pfu (Qiagene). Two PCR products of *RCIFS* and *RCPR* were ligated *via* PCR annealing, digested with NotI, and then cloned into corresponding sites of pGEX 5X-3 (Amersham Biotech, U.S.A.). The resultant construct was designated *RCIFS–RCPR*. The fusion gene of *RCIFS* and *CPR* from *Catharanthus roseus* (C*CPR*) was also constructed as described above, with 5'-TACCCGATCCGGAAGATGTCGACCCAGAGGAAAGGA GTTT-3' as the *RCIFS* reverse primer (underlined Gly-Ser-Thr linker sequence), 5'-TAAACTCCTTTCCTCTGGGTCGACATCTTCCGGAT CGGGT-3' as the *CCPR* (GenBank Accession No. X69791.1) forward primer (underlined Gly-Ser-Thr linker sequence), and ATGCGGCCGC TCACCAGACATCTCGGA as the *CCPR* reverse primer (underlined NotI site). The resulting construct was designated *RCIFS–CCPR*.

Biotransformation with Whole Cells Expressing *RCIFS–RCPR* and *RCIFS–CCPR*, Respectively

E. coli transformants harboring either *RCIFS–RCPR* or *RCIFS–CCPR* were grown for the seed culture in LB medium containing 50 μg/ml of ampicillin. The seed culture was inoculated in 50 ml of fresh LB containing 50 μg/ml of ampicillin. The culture was grown until absorbance at 600 nm reached 0.6. At this point, IPTG was added at a final concentration of 0.1 mM, and the transformant was grown for over 12 h with shaking at 200 rpm at 18°C. The cells were harvested *via* centrifugation and resuspended in new LB medium containing 50 μg/ml of ampicillin and 0.1 mM IPTG, respectively. Substrate (naringenin) was added to a final concentration of 80 μM, and the mixture was incubated for 48 h at 28°C. The supernatant was then collected several times and extracted twice with ethyl acetate. Finally, the ethyl acetate extract was evaporated in a speed vacuum and dissolved in 80 μl of dimethyl sulfoxide (DMSO).

The production of genistein using an *E. coli* transformant in different culture media such as LB (Luria–Bertani Broth), LB plus 1% glucose, M9, M9 plus 1% glucose, M9 plus 2% glucose, TB (Terrific Broth), and TB plus 2% glucose was conducted as follows. The induction of the *RCIFS–RCPR* fusion gene in *E. coli* was conducted as described above. After the cells were harvested, the same quantity of cells was resuspended in each medium. Eighty μM of naringenin was added and the mixture was incubated for 12 h at 28°C.

HPLC Analysis of Reaction Products

The reaction products were analyzed using a Varian high performance liquid chromatography apparatus (Walnut Creek, CA, U.S.A.) 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). For analytical scale, the mobile phase consisted of 0.1% formic acid (pH 3.0), programmed as follows: 20% acetonitrile at 0 min, 45% acetonitrile at 10 min, 60% acetonitrile at 20 min, 20% acetonitrile at 30 min, and 20% acetonitrile at 40 min. The flow rate was 1 ml/min and UV detection was dually monitored at 260 nm and 290 nm.

Results and Discussion

RCIFS is a cytochrome P450. Cytochrome P450 harbors a membrane anchorage domain that is hydrophobic and renders the protein insoluble [15]. Secondary structure analysis of RCIFS using the MLRC secondary structure prediction program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_mlrc.html) revealed a helix motif at the first 21 amino acids of the N-terminal region, which has been identified as a membrane anchor sequence. The first 21 amino acids at the N-terminal region of the *RCIFS* were eliminated in order to achieve the functional expression of *IFS* in *E. coli*. The first codon of the truncated *IFS* gene was subsequently exchanged into ATG. Finally, the stop codon of *RCIFS* was removed and then a Gly-Ser-Thr linker sequence was introduced in order to fold the two proteins separately. Next, *RCPR* was fused to this modified gene. Similar to the case of *RCIFS*, the membrane binding domain of RCPR (the first 50 amino acids) was removed and a Gly-Ser-Thr linker sequence was introduced into the N-terminal region. These two modified genes were then fused *via* a polymerase chain reaction and subcloned into pGEX. The resultant construct was verified *via* DNA sequencing. The construction strategy of the fusion chimera is described in Fig. 1. Another CPR from *C. roseus* was also connected to the modified *RCIFS via* the same strategy. The final constructs were designated *RCIFS–RCPR* and *RCIFS–CCPR*. The two constructs were transformed separately into *E. coli* BL21 (DE3).

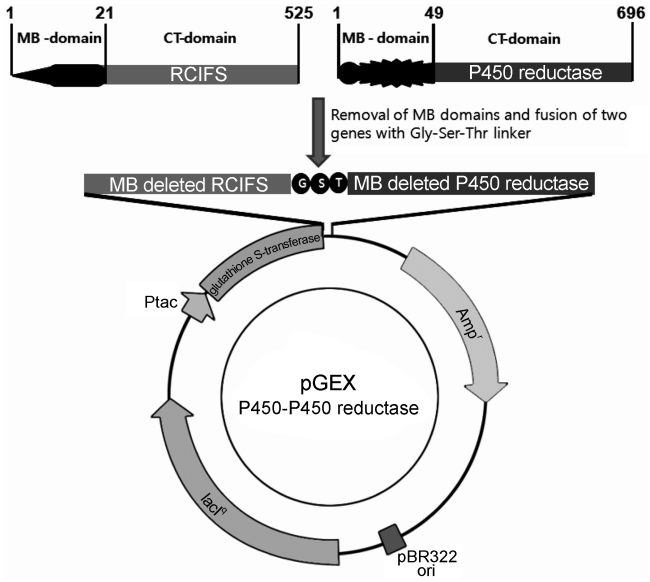

Fig. 1. Schematic diagram representing the construction of a functional fusion of RCIFS (isoflavone synthase from red clover) and RCPR (Cytochrome P450 reductase from *Oryza sativa*).
MB-domain: membrane binding domain, CT-domain; catalytic domain; GST: Glycine-Serine-Threonine linker.

The biotransformation of naringenin with *E. coli* transformants was conducted. After 24 h, the reaction product from *E. coli* harboring *RCIFS–RCPR*, as well as that from *E. coli* harboring *RCIFS–RCPR*, yielded new peaks (P1 and P2 in Fig. 2C) when analyzed *via* HPLC. P2 evidenced a retention time identical to that of authentic genistein (Fig. 2C). Additionally, the UV spectrum of the reaction product was indistinguishable from that of authentic genistein. The molecular mass of P2 was 270 Da, which is identical to that of genistein. P1 was assumed to be a reaction intermediate, 2,5,7,4'-tetrahydroxyisoflavanone, which appeared during the conversion of naringenin to genistein [1]. These results showed that both of the *E. coli* transformants converted naringenin to genistein. However, *E. coli* harboring *RCIFS–CCPR* converted naringenin into genistein less effectively than *E. coli* harboring *RCIFS–RCPR* (data not shown). It appears likely that the CPR from *C. roseus* did not effectively transfer electrons from NADPH to RCIFS. It is known that the capacity of CPR to transfer the electron to the corresponding P450 is crucial for the catalytic efficiency of CPR [12, 16, 17].

In an effort to maximize the production of genistein in the *E. coli* transformant, we tested six culture media: LB (Luria–Bertani Broth), LB plus 1% glucose, M9, M9 plus 1% glucose, M9 plus 2% glucose, TB (Terrific Broth), and TB plus 2% glucose. After the induction of genes in the *E. coli* transformants, the cells were harvested and resuspended in the above-mentioned medium. Among the tested culture media, LB was determined to be the most efficient culture medium for genistein production (Fig. 3). The addition of glucose to the medium conspicuously inhibited genistein production. Genistein production in TB or M9 was measured at about 72% and 35% of that observed in LB. Although the initial amount of cell concentration was identical in six different media, the final cell density of each medium differed; TB medium was best and M9 was worst. However, different cell growth was not the only factor in the maximum

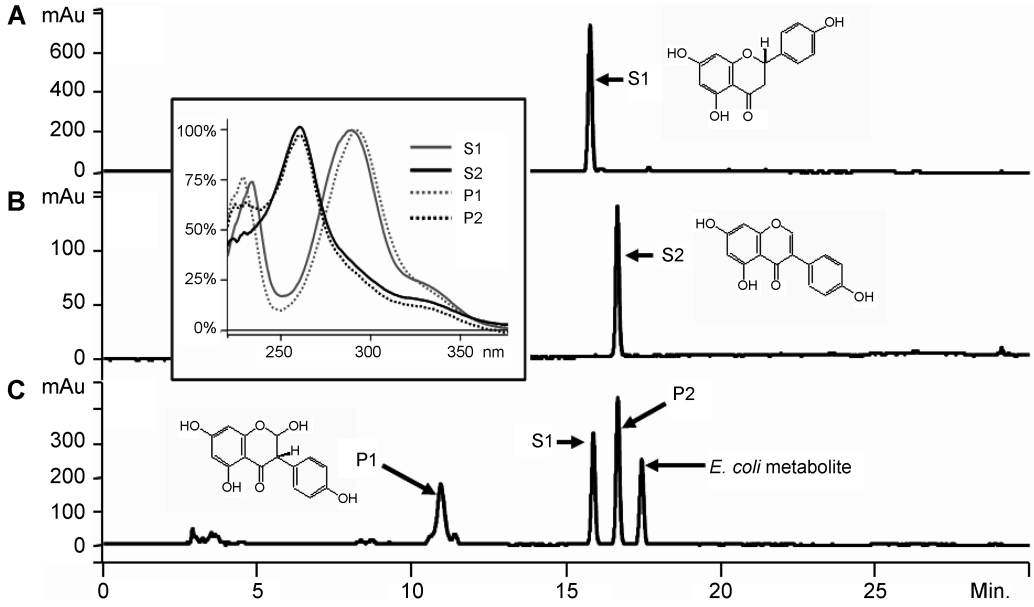

Fig. 2. HPLC analysis of reaction product generated by *E. coli* harboring *RCIFS–RCPR*.
A. Authentic naringenin; **B**. Authentic genistein; **C**. Reaction products of naringenin with *E. coli* harboring *RCIFS–RCPR* (P1, 2,5,7,4'-tetrahydroxyisoflavanone [1]; P2, genistein).

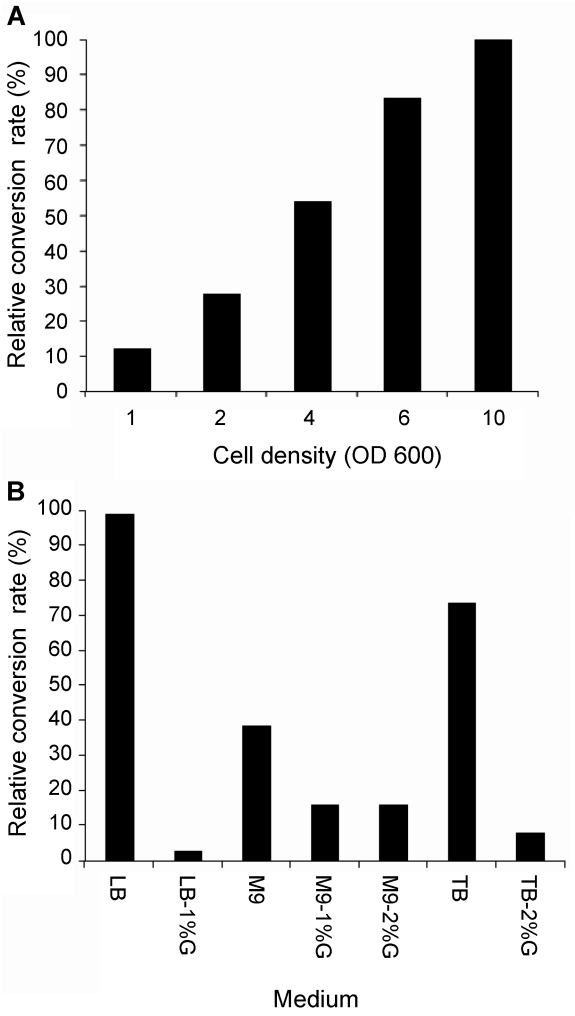

Fig. 3. Determination of cell density (**A**) and optimum culture medium (**B**) for maximum production using *E. coli* harboring *RCIFS–RCPR*.

production of genistein, as LB is not the optimal medium for the production of cell growth.

We also assessed the effects of initial cell density with regard to genistein production. After the induction of the fusion gene in *E. coli*, cell densities were adjusted to 1, 2, 4, 6, and 10 at 600 nm using LB containing 50 μg/ml of ampicillin. *E. coli* harboling either *RCIFS-RCPR* or *RCIFS-CCPR* increased genistein production when the cell density was increased. Although the most effective cell density at OD_{600} was 10, it was noted that *E. coli* metabolites were quite high at this cell density. Thus, we elected to utilize a cell density at OD_{600}=6, at which the purification of genistein proved easier owing to the low concentration of *E. coli* metabolites.

With the culture medium and under optimized cell density conditions, the production of genistein using *E. coli* IR or IC was monitored. Eighty μM of naringenin, which is a mixture of two enantiomers (*R* and *S* forms of naringenin),

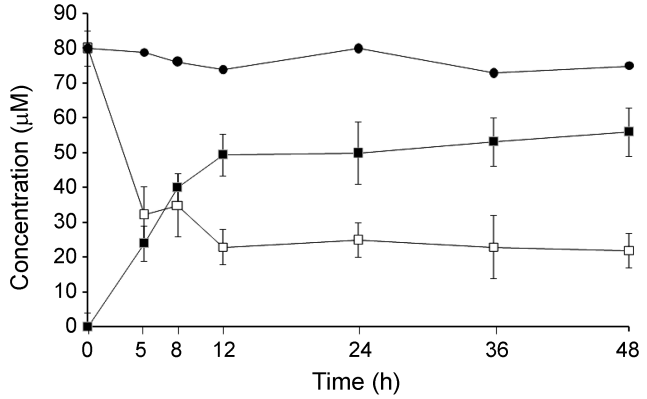

Fig. 4. Biotransformation of naringenin to genistein using *E. coli* expressing *RCIFS–RCPR*.
After 20 h of induction at 18°C, the cells were harvested and resuspended with 25 ml of fresh LB medium containing 50 μg/ml of ampicillin. Cell density was adjusted at OD_{600}=6. Eighty μM of naringenin was added and the culture was incubated at 28°C. The reactant was periodically harvested and was extracted twice with an equal volume of ethyl acetate, and the reaction products were quantified *via* HPLC. (Filled circle, naringenin in *E. coli* harboring vector pGEX; filled square, genistein in *E. coli* harboring *RCIFS–RCPR*; open square, naringein in *E. coli* harboring *RCIFS–RCPR*)

was added to the *E. coli* transformants after the induction of either gene. As shown in Fig. 4, RCIFS–RCPR was able to produce 56 μM of genistein. However, *E. coli* harboring only vector did not produce genistein, and the concentration of naringenin remained unchanged.

In the current study, the yield of genistein by transgenic *E. coli* was calculated as approximately 16 mg per liter. However, transgenic yeast harboring IFS was measured at 5.9 mg per liter [8]. The observed difference in yield might be the result of (1) different levels of IFS expression in *E. coli* and *S. cerevisiae* and/or (2) the partnership of IFS and CPR, because the previous study utilized *S. cerevisiae* cytochrome CPR rather than plants. The approach we utilized could be applicable to the expression of other P450s from plants for the definition of substrates or the modification of chemicals including flavonoids or antibiotics.

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