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REVIEW PAPER

Masafumi Kaneko · Eui Il Hwang · Yasuo Ohnishi
Sueharu Horinouchi

Heterologous production of flavanones in *Escherichia coli*: potential for combinatorial biosynthesis of flavonoids in bacteria

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Abstract Chalcones, the central precursor of flavonoids, are synthesized exclusively in plants from tyrosine and phenylalanine via the sequential reaction of phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate:coenzyme A ligase (4CL) and chalcone synthase (CHS). Chalcones are converted into the corresponding flavanones by the action of chalcone isomerase (CHI), or non-enzymatically under alkaline conditions. PAL from the yeast *Rhodotorula rubra*, 4CL from an actinomycete *Streptomyces coelicolor* A3(2), and CHS from a licorice plant *Glycyrrhiza echinata*, assembled as artificial gene clusters in different organizations, were used for fermentation production of flavanones in *Escherichia coli*. Because the bacterial 4CL enzyme attaches CoA to both cinnamic acid and 4-coumaric acid, the designed biosynthetic pathway bypassed the C4H step. *E. coli* carrying one of the designed gene clusters produced about 450 μg naringenin/l from tyrosine and 750 μg pinocembrin/l from phenylalanine. The successful production of plant-specific flavanones in bacteria demonstrates the usefulness of combinatorial biosynthesis approaches not only for the production of various compounds of plant and animal origin but also for the construction of libraries of "unnatural" natural compounds.

Keywords Combinatorial biosynthesis · Flavonoid
Flavanone · Chalcone · Metabolic engineering ·
4-Coumarate/cinnamate:CoA ligase

Dedicated to Professor Sir David Hopwood.

M. Kaneko · E. I. Hwang · Y. Ohnishi · S. Horinouchi (✉)
Department of Biotechnology ,
The University of Tokyo,
Graduate School of Agriculture and Life Sciences,
Bunkyo-ku, 113–8657 Tokyo, Japan
E-mail: asuhori@mail.ecc.u-tokyo.ac.jp
Tel.: +81-3-58415123
Fax: +81-3-58418021

Introduction

Flavonoid-derived natural products in plants are well known floral pigments [13, 46, 53, 56] that also function as pollen fertility factors [11, 19, 45, 62, 64], signal molecules for beneficial plant-microbe symbiosis in the rhizosphere [52, 63], and antimicrobial defense compounds [24, 28, 37, 65, 68]. Following reports of flavonoid compounds having cancer chemopreventive, antioxidant and antiasthmatic activities, there has been an explosion of interest in their use as health-promoting components of the human diet [1, 7, 10, 12, 15, 18, 21, 23, 29, 40, 42–44, 58, 59, 66]. These phenylpropanoid and flavonoid biosynthetic enzymes are therefore attractive metabolic engineering targets of processes to enhance or initiate the production of economically desirable traits or compounds. The phenylpropanoid and flavonoid biosynthetic pathways and their regulation have been the subjects of many studies [16, 20, 26, 55, 67]. Recent advances in the regulation of these pathways and the biochemistry of their specific enzymes and enzyme complexes have opened up strategies to increase flavonoid biosynthesis by genetic engineering [8, 9, 25].

In the plant phenylpropanoid pathway (Fig. 1), phenylalanine ammonia-lyase (PAL), which deaminates phenylalanine to yield cinnamic acid, is the first enzyme in the general phenylpropanoid pathway. Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (C4H) to 4-coumaric acid, which is then activated to 4-coumaroyl-CoA by the action of 4-coumarate:coenzyme A ligase (4CL). Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with 4-coumaroyl-CoA to yield naringenin chalcone, the precursor of a large number of flavonoids. Naringenin chalcone is converted to naringenin by chalcone isomerase (CHI) or non-enzymatically in vitro under alkaline conditions [47].

The production of flavonoids by genetically engineered bacteria has not yet been reported, although the

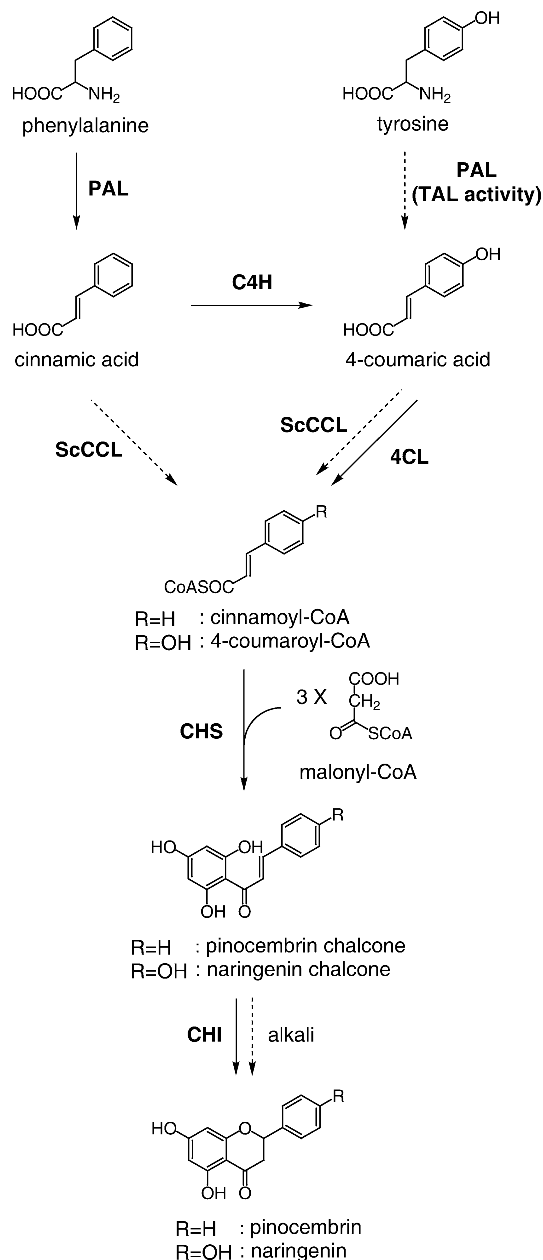

Fig. 1 Flavanone biosynthetic pathway in plants. The *dotted arrows* represent the expected bypass pathway for combinatorial biosynthesis of flavanones in *Escherichia coli*. *PAL* Phenylalanine ammonia-lyase, *TAL* tyrosine ammonia-lyase, *C4H* cinnamate-4-hydroxylase, *4CL* 4-coumarate:coenzyme A ligase, *ScCCL* 4-coumarate/cinnamate:coenzyme A ligase from *Streptomyces coelicolor* A3(2), *CHS* chalcone synthase, *CHI* chalcone isomerase

heterologous expression of phenylpropanoid biosynthetic enzymes in bacteria was described previously [2, 31, 41, 69]. One of the barriers to the production of these compounds is the difficulty in expressing active C4H, which could not be efficiently expressed in bacteria due to its instability and the lack of a specific cytochrome P-450 reductase [51]. We recently discovered a 4CL in the gram-positive, filamentous bacterium *Streptomyces coelicolor* A3(2) that can activate cinnamic acid to cinnamoyl-CoA, in addition to 4-coumaric acid to 4-coumaroyl-CoA [35]. Using the 4CL enzyme would bypass the C4H step for the production of pinocembrin chalcone from phenylalanine via the phenylpropanoid pathway. In this review article, we briefly describe the phenylpropanoid pathway in plants and the successful production of flavanones in *Escherichia coli* by inserting the bacterial 4-coumarate/cinnamate:CoA ligase gene within an artificial gene cluster for combinatorial biosynthesis. Successful heterologous production of plant-specific flavanones will allow a variety of flavonoids to be produced in bacteria by further genetic engineering, similar to the excellent results reported for the biosynthesis of polyketides [49, 50, 70].

Phenylpropanoid pathway in plants

Phenylalanine ammonia-lyase

As the first step in the phenylpropanoid pathway, PAL (EC 4.3.1.5) catalyzes the elimination of ammonia from phenylalanine to yield cinnamic acid (Fig. 1). PAL activity has been found in some fungi, bacteria, and in all higher plants but not in animals. In plants, PAL has been extensively studied for its roles in the formation of lignins, isoflavonoids, and other secondary metabolites. In addition, tyrosine ammonia-lyase (TAL) catalyzes the formation of 4-coumaric acid from tyrosine. Because some PALs have TAL activity [39, 54, 57], both phenylalanine and tyrosine are expected to be precursors in the combinatorial biosynthesis of flavonoids in bacteria.

Cinnamate-4-hydroxylase

Cinnamic acid is hydroxylated by the action of C4H (EC 1.14.13.11). This enzyme is a membrane-bound cytochrome P-450 hydroxylase that requires molecular oxygen and a reducing equivalent from NADPH delivered via cytochrome P-450 reductase [31, 51]. Therefore, efficient expression of a C4H gene in bacteria requires simultaneous expression of the specific P-450 reductase gene.

4-Coumarate:coenzyme A ligase

4-coumarate:CoA ligase (EC 6.2.1.12) catalyzes the conversion of 4-coumarate (4-hydroxycinnamate) and other substituted cinnamates, such as caffeate (3,4-dihydroxycinnamate) and ferulate (3-methoxy-4-hydroxycinnamate), into the corresponding CoA thiol esters, which are used for the biosynthesis of numerous phenylpropanoid-derived compounds, such as lignins, lignans, suberins, flavonoids, isoflavonoids, and various small phenolic compounds. 4CL-catalyzed CoA ester formation takes place via a two-step reaction. During the first step, 4-coumarate and ATP form a coumaroyl-adenylate

intermediate with the simultaneous release of pyrophosphate. In the second step, the coumaroyl group is transferred to the sulfhydryl group of CoA, and AMP is released [3, 36]. The mechanism of formation of an adenylate intermediate is common among a number of enzymes with divergent functions, including luciferases, fatty acyl-CoA ligases, acetyl-CoA ligases, and specialized domains within peptide synthetase multi-enzymes. Despite their low overall amino acid sequence identity, the similar reaction mechanisms of these enzymes and the presence of conserved peptide motifs were used as criteria to classify them in a superfamily of adenylate-forming enzymes [22]. The relationship of 4CL to other adenylate-forming enzymes was substantiated recently by functional analysis of key 4CL amino acid residues that are conserved in other adenylate-forming enzymes [60].

Chalcone synthase

Chalcone synthase (EC 2.3.1.74) is a plant-specific polyketide synthase that uses a starter CoA-ester, typically 4-coumaroyl-CoA, derived from the phenylpropanoid pathway. It catalyzes three condensation reactions with malonyl-CoA and folds the resulting tetraketide intermediate into a new aromatic ring system [17, 33, 38]. After initial capture of the 4-coumaroyl moiety, each subsequent condensation step begins with decarboxylation of malonyl-CoA at the CHS active site, and the resulting acetyl-CoA carboanion then serves as the nucleophile for chain elongation. Ultimately, these reactions generate a tetraketide intermediate that cyclizes by a Claisen condensation into a hydroxylated aromatic ring system [17]. CHS supplies 4,2′,4′,6′-tetrahydroxychalcone to downstream enzymes that synthesize a wide variety of flavonoids, such as phytoalexins and anthocyanin pigments.

Chalcone isomerase

In the last stages of the biosynthesis of flavanone, chalcone isomerase (EC 5.5.1.6) catalyzes the intramolecular cyclization of 4,2′,4′,6′-tetrahydroxychalcone (chalcone) and 6-deoxychalcone (4,2,4-trihydroxychalcone), both derived from the upstream enzyme CHS, into (2*S*)-naringenin (5,7,4-trihydroxyflavanone) and (2*S*)-5-deoxyflavanone (7, 4-dihydroxyflavanone), respectively [27, 48]. Although chalcones spontaneously cyclize in alkaline solution to produce an enantiomeric mixture of flavanones, CHI directs formation of biologically active (2*S*)-flavanones [4, 5, 34].

4-Coumarate/cinnamate:coenzyme A ligase from *S. coelicolor* A3(2)

The filamentous, soil-living, gram-positive bacterial genus *Streptomyces* is characterized by the ability to produce a wide variety of secondary metabolites, including antibiotics, and by complex morphological differentiation culminating in sporulation [30]. *S. coelicolor* A3(2) has been most extensively and intensively characterized among *Streptomyces* by the research group of D.A. Hopwood, and recently the whole genome has been sequenced (http://www.sanger.ac.uk/Projects/S_coelicolor) [6]. In the database, a gene (SCD10.15) encoding a 522-amino-acid protein has been annotated as a 4CL gene. The protein has higher sequence similarity to plant 4CLs than to bacterial acyl-CoA ligases; it shows 44% identity and 58% similarity to *Arabidopsis* At4CL2. This is the first bacterial protein that shows end-to-end sequence similarity to plant 4CLs over 40% identity. The recombinant protein, expressed in *E. coli*, had distinct 4CL activity, but its substrate specificity was unique; the enzyme efficiently converted cinnamate, which is a very poor substrate for plant 4CLs [35]. The enzyme was therefore named ScCCL, for *S. coelicolor* A3(2) cinnamate:CoA ligase (Fig. 2). The 4-coumarate/cinnamate:CoA ligase activity of the enzyme is useful for the production of flavanones by combinatorial biosynthesis in bacteria in that it can bypass the C4H step, which is apparently difficult to express in bacteria, as described above.

Flavanone fermentation in bacteria

For the purpose of production of flavanones in *E. coli*, an artificial gene cluster was constructed that contained three genes of heterologous origins: *PAL* from a yeast *Rhodotorula rubra*, *4CL* from an actinomycete *S. coelicolor* A3(2), and *CHS* from a licorice plant *Glycyrrhiza echinata* (Fig. 3) [32]. The chalcones were expected to be converted in vitro to the corresponding flavanones by raising the pH of the culture broth to 9. Because PAL uses phenylalanine and tyrosine as substrates, 4CL attaches CoA to both cinnamate and 4-coumarate, and CHS forms chalcones from cinnamate-CoA and 4-coumarate-CoA, the respective artificial gene cluster was

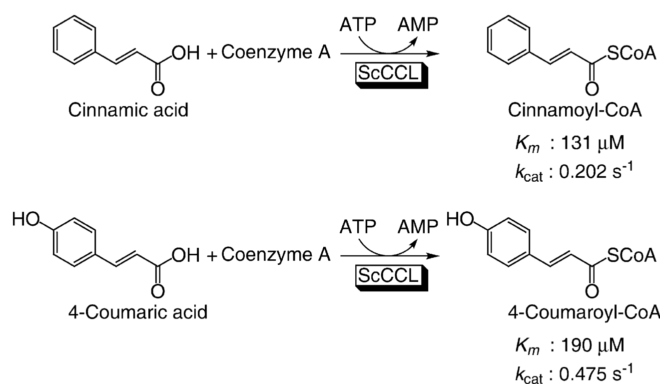

Fig. 2 Substrate specificity of 4-coumarate/cinnamate:CoA ligase (ScCCL) from *S. coelicolor* A3(2). ScCCL is the only enzyme so far isolated that ligates CoA to cinnamic acid

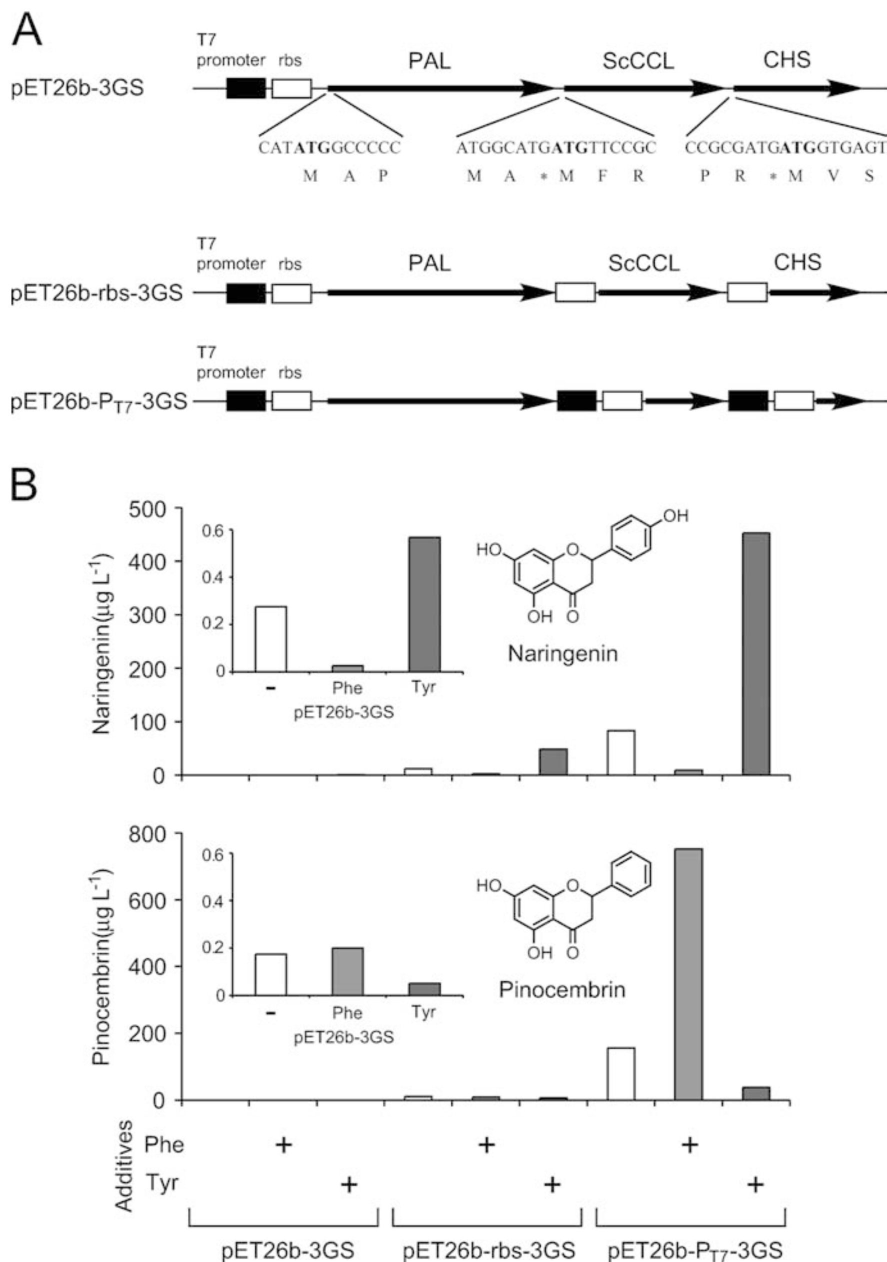

Fig. 3 Organization of the artificial gene clusters used for production of flavanones in *E. coli*. **A** In pET26b-3GS, *PAL* from a yeast, *ScCCL* from an actinomycete, and *CHS* from a licorice plant were placed under the control of the T7 promoter and ribosome-binding sequence in pET26b. The initiation codons of *ScCCL* and *CHS* overlap the termination codon of the preceding genes. In pET26b-rbs-3GS, the three genes (all with the ribosome-binding sequence at appropriate positions) are co-transcribed from the T7 promoter in front of *PAL*. In pET26b-P_{T7}-3GS, all three genes contain their own T7 promoter and ribosome-binding sequences. **B** Accumulation of naringenin and pinocembrin in the culture broth of *E. coli* harboring the artificial gene clusters

expected to direct the synthesis of pinocembrin from phenylalanine and naringenin from tyrosine (Fig. 1). We first generated a gene cluster in which *PAL*, *4CL* and *CHS* genes were placed in this order under the control of the T7 promoter (P_{T7}) and the ribosome-binding sequence in the pET vector. The translational initiation codons of *4CL* and *CHS* overlapped with the termination codons of the preceding genes. This type of gene organization is often found in bacterial gene clusters, such as those for antibiotic and xenobiotic biosynthesis. Plasmid pET26b-3GS carrying the gene cluster, however, caused *E. coli* BL21 (DE3) to produce only a very small amount of pinocembrin and naringenin, even when the amino acid precursors phenylalanine and tyrosine were added; the bacteria produced 0.2 μg pinocembrin/l and 0.57 μg naringenin/l , when 2 mM of each of the amino acid precursors was supplied. By contrast, 4-coumaric acid and cinnamic acid accumulated in large amounts, about 9 mg/l and 12 mg/l, respectively.

Accumulation of 4-coumaric acid and cinnamic acid suggested that *4CL* and *CHS* were not expressed efficiently. We therefore constructed pET26b-rbs-3GS, in which the three genes were transcribed by a single P_{T7} in front of *PAL*, and each of the three contained rbs at appropriate positions and pET26b-P_{T7}-3GS, in which all three genes contained both P_{T7} and a ribosome-binding sequence (Fig. 3). Placement of a ribosome-binding sequence in front of each gene (plasmid pET26b-rbs-3GS) enhanced the yields of pinocembrin and naringenin by about 45-fold and 85-fold, respectively. Consistent with this, SDS-PAGE of a cell-lysate

prepared from *E. coli* harboring pET26b-rbs-3GS revealed the presence of large amounts of soluble PAL, 4CL and CHS. Furthermore, large amounts of flavanones (about 750 μg pinocembrin/l and 450 μg naringenin/l) were produced. These findings show the importance of efficient transcription from the T7 promoter and efficient translation from the ribosome-binding sequence.

The yields of flavanones were still low. We expect that an increase in the amount of a precursor, malonyl-CoA, which is present at 4–90 μM (0.01–0.23 nmol/mg dry weight) in *E. coli* under normal cultural conditions [6], by overexpression of the acetyl-CoA carboxylase gene [14] would lead to enhancement of the yields. A fermentation condition to remove the ammonia produced by the action of PAL would also increase the yields. The use of a mutant strain of *Corynebacterium glutamicum*, e.g., a phenylalanine or tyrosine fermenter, would release us from the need to provide amino acids to the culture. Concerning the host cells, yeast and fungi, such as *Saccharomyces cerevisiae* and *Aspergillus oryzae*, may be useful for functional expression of P-450 genes that are required for the further conversion of flavanones.

Concluding remarks

The genomes of *Streptomyces* are useful sources of genes coding for a variety of functions, since these bacteria produce a variety of unique secondary metabolites. Genome sequencing of additional *Streptomyces* species could very well lead to the discovery of enzymes that are applicable to the bioconversion and combinatorial biosynthesis of useful compounds. The production of plant-specific flavanones in *E. coli* serves as an example of the usefulness of this type of metabolic engineering. We believe that it can be applied to the production of a variety of compounds of plant and animal origin in bacteria. In addition, the this approach together with combinatorial biosynthesis will allow the construction of libraries of "unnatural" natural products.

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