J. Antibiot. 61(12): 709–728, 2008

REVIEW ARTICLE

THE JOURNAL OF ANTIBIOTICS

Combinatorial Biosynthesis of Non-bacterial and Unnatural Flavonoids, Stilbenoids and Curcuminoids by Microorganisms

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Received: August 1, 2008 / Accepted: October 14, 2008

Abstract One of the approaches of combinatorial biosynthesis is combining genes from different organisms and designing a new set of gene clusters to produce bioactive compounds, leading to diversification of both chemical and natural product libraries. This makes efficient use of the potential of the host organisms, especially when microorganisms are used. An *Escherichia coli* system, in which artificial biosynthetic pathways for production of plant-specific medicinal polyketides, such as flavonoids, stilbenoids, isoflavonoids, and curcuminoids, are assembled, has been designed and expressed. Starting with amino acids tyrosine and phenylalanine as substrates, this system yields naringenin, resveratrol, genistein, and curcumin, for example, all of which are beneficial to human health because of their wide variety of biological activities. Supplementation of unnatural carboxylic acids to the recombinant *E. coli* cells carrying the artificial pathways by precursor-directed biosynthesis results in production of unnatural compounds. Addition of decorating or modification enzymes to the artificial pathway leads to production of natural and unnatural flavonols, flavones, and methylated resveratrols. This microbial system is promising for construction of larger libraries by employing other polyketide synthases and decorating enzymes of various origins. In addition, the concept of building and expressing artificial biosynthetic pathways for production of non-bacterial and unnatural compounds in microorganisms should be successfully applied to production of not only plant-specific polyketides but also many other useful compound classes.

Keywords combinatorial biosynthesis, metabolic engineering, medicinal polyketides from plants, precursor-directed biosynthesis, flavonoids, stilbenoids, curcuminoids

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1. Introduction

Combinatorial biosynthesis is a new tool in the generation of novel natural products and for production of rare and expensive natural products. The concept of combinatorial biosynthesis involves genetically engineered enzymes that are used to make novel, complex compounds as drug development candidates. Two types of enzyme systems operate like an assembly line, produce a diverse group of natural products, and have proven to be useful for generation of compound libraries. These systems are composed of multiple modules, in which an individual module consists of either a polyketide synthase (PKS) or a nonribosomal peptide synthetase (NRPS). Each module has a specific set of catalytic domains that determine the structure of the metabolic product. The feasibility of combinatorial biosynthesis, *i.e.*, the genetic manipulation of PKS biosynthetic pathways, was first demonstrated by Hopwood *et al.* [1]. They introduced some or all of the actinorhodin biosynthetic pathway in *Streptomyces coelicolor* A3(2) into a medermycin-producing *Streptomyces* sp. AM-7161 and dihydrogranaticin-producing *Streptomyces violaceoruber*. The AM-7161 transformant produced a large amount of a new, hybrid compound named mederrhodin A, which carried an additional hydroxy group characteristic of actinorhodin. The *S. violaceoruber* transformant also produced a new, hybrid compound dihydrogranatirhodin, which had both the actinorhodin and dihydrogranaticin configurations in the molecule. This is a good example of combinatorial biosynthesis, in which the combination of the product of one species and the enzymes of another species yields a desired product. An important example of combinatorial biosynthesis of this type was the development of erythromycin analogues; McDaniel *et al.* [2] used sets of genes from different biosynthetic pathways in different combinations to generate libraries of hybrid structures derived from 6-deoxyerythronolide B, the aglycon of erythromycin, which is assembled on a type I modular PKS.

Because of the rapid development in molecular biological techniques and the explosive accumulation of the genome information from a variety of organisms [3, 4], combinatorial biosynthesis now has a wider definition. Bioinformatic prediction of the catalytic property of a single gene product and novel chemical entities directed by a gene cluster in the huge genome databases facilitates combining metabolic pathways to generate artificially

designed biosynthetic pathways for production of targeted compounds in different organisms. As a consequence the host organisms provide precursors from their own primary and secondary metabolism that are converted to the expected secondary product through the expression of foreign genes. Our success in fermentative production of plant-specific polyketides by *E. coli* carrying an artificially assembled phenylpropanoid pathway was the first example to show that a nearly complete biosynthetic pathway in plants was established in a heterologous microorganism for production of flavanones from the amino acid precursors, phenylalanine and tyrosine [5, 6]. Later, Yan *et al.* [7] produced 5-deoxyflavanones in *E. coli* and Beekwilder *et al.* [8] produced a natural raspberry ketone in *E. coli*. The approach of this type of combining genes from different organisms and designing a gene cluster to produce a bioactive compound leads not only to diversification of both chemical and natural product libraries but also to efficient use of the potential of the host organisms, particularly when microorganisms are used as the hosts. The rapid growth of microorganisms in inexpensive media, the ease of strain improvement by conventional mutagenesis, and screening have made them the central hosts of biotechnology. Taken together, established techniques in molecular biology and fermentation of microorganisms, explosive accumulation of genome information of a variety of microorganisms, and the development of a "clean" host on the basis of the genome information increasingly make this approach practicable and important for diversification of natural products.

Assembly of the genes to make up artificial biosynthetic pathways is essential when we produce plant-specific flavonoids, including unnatural compounds, in microorganisms. The enzyme included as one of the steps in the phenylpropanoid pathway is a type III PKS, which is the simplest dimer form among the three PKS types I, II, and III [9∼11]. Because the catalytic mechanisms regarding loading of the starter substrate, extension of the extender substrate, and cyclization and release of the extended product in a single, multifunctional catalytic pocket formed by a homodimer of the ketosynthase are not fully understood, it is not possible to use type III PKSs and their mutant enzymes to produce a diverse group of natural products, in contrast to type I modular PKSs which consist of multiple, interchangeable modules and which operate like an assembly line. Instead, different type III PKSs showing various substrate specificity and cyclization modes can be used as one of the steps in artificially assembled pathways to produce a diverse group of flavonoids and their related compounds in microorganisms. Screening of type III PKSs that possess novel catalytic properties is therefore important for further diversification of the compounds produced by our strategy.

In this review, I summarize the production of natural and unnatural flavonoids (C_6–C_3–C_6), stilbenoids (C_6–C_2–C_6) and curcuminoids (C_6–C_7–C_6) by the host cell of *E. coli* by means of assembly and expression of artificial biosynthetic pathways. This project was started in our laboratory after a type III PKS, named RppA [12, 13], was for the first time discovered from the Gram-positive bacterial genus *Streptomyces* and its catalytic properties were elucidated. In addition, the presence of a cinnamate/4-coumarate:CoA ligase in *Streptomyces* [14], which is also included as a step in the phenylpropanoid pathway in plants, strongly prompted us to initiate the project. The presence of an enormous number of genes for secondary metabolite formation and modifications of exogenous substrates enabled the design of a project to produce plant-specific flavonoids in microorganisms [15]. Although this review focuses on our study of the microbial production of plant-specific polyketides, I will briefly touch some outgrowths derived from this project.

2. *In Silico* Survey of Type III PKSs and a Cinnamate/4-coumarate:CoA Ligase

2-1. RppA, a Type III PKS for Synthesis of a Naphthalene Derivative

During our study of the A-factor regulatory cascade in streptomycin-producing *Streptomyces griseus* [15, 16], I observed a colony producing a red-brown pigment among the transformants that carried a library of the DNA fragments from *S. griseus* on a plasmid vector [12]. The colony carried a DNA fragment containing two genes, one encoding a protein that showed end-to-end similarity to chalcone synthases, belonging to the type III PKS family, and the other encoding a cytochrome P450-like protein. *In vivo* and *in vitro* analysis of the chalcone synthase-like protein, named RppA (red-brown pigment production), established that it utilizes malonyl-CoA as the starter and extender, carries out four successive extensions and releases the resulting pentaketide to cyclize to form 1,3,6,8-tetrahydroxynaphthalene (THN) (Fig. 1). This was the first discovery of a functional type III PKS from the bacterial world [13, 17, 18]. THN auto-oxidizes to form flaviolin, a red-brown pigment, which explains why the originally isolated transformant carrying *rppA* was red-brown. The cytochrome P450-like enzyme as a partner of RppA was found to convert the THN into 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone (HPQ) by oxidative biaryl coupling [19]. HPQ readily autopolymerizes to form

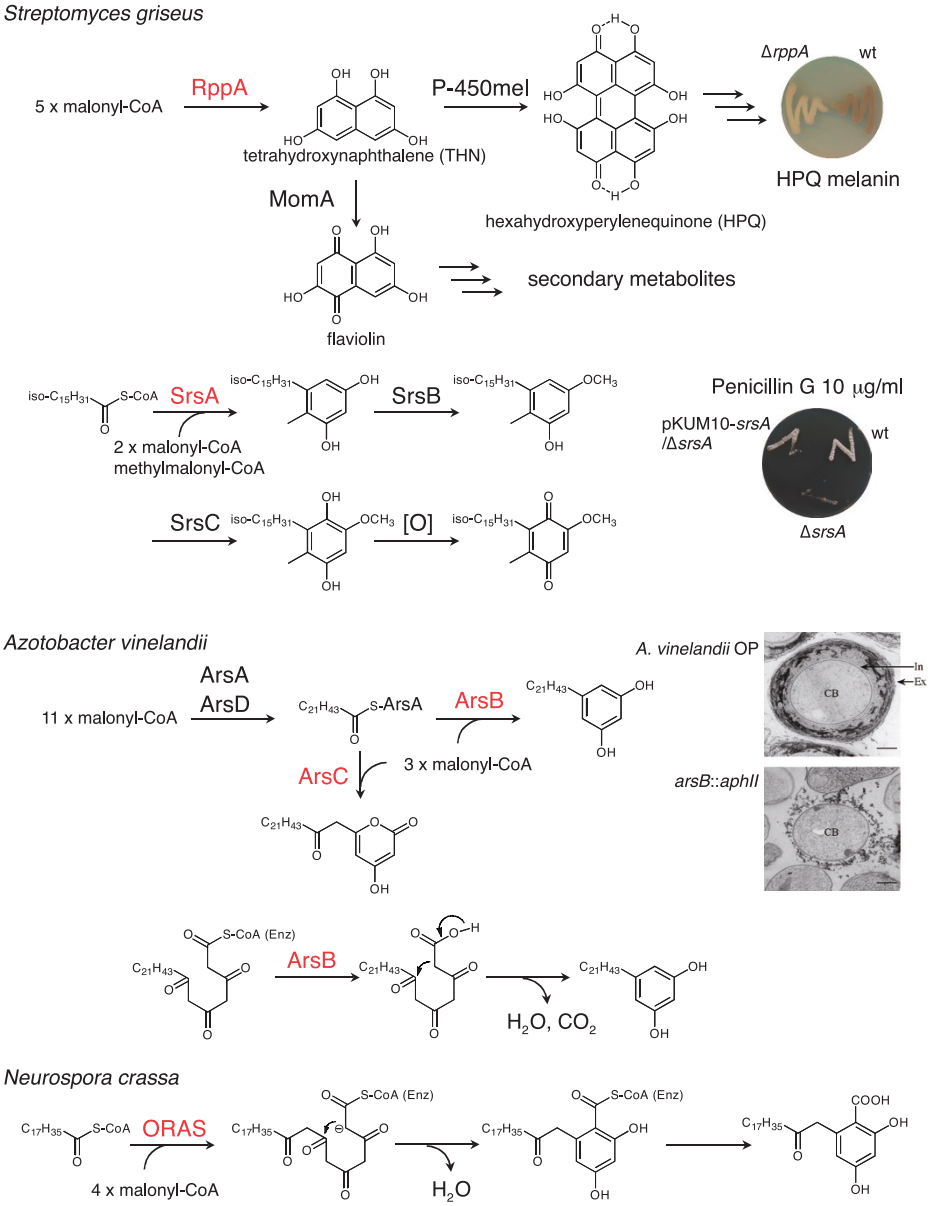

Fig. 1 Catalytic properties of some type III polyketide synthases of microbial origins.

RppA in *S. griseus*, the first type III PKS found in the bacterial world, synthesizes THN from five molecules of malonyl-CoA. The THN formed is converted either to HPQ by a partner of RppA, which finally auto-oxidizes to form HPQ melanin, or to flaviolin by MomA, which is further converted to secondary metabolites. The photograph shows HPQ melanin production by the wild-type (wt) *S. griseus* strain and an "albino" phenotype of mutant Δ*rppA* [13]. On the other hand, SrsA synthesizes phenolic lipids, which is then modified by its partners, SrsB and SrsC. The phenolic lipids, perhaps integrated in the membrane, confer penicillin resistance on *S. griseus*. The wt *S. griseus* strain grows on medium containing 10 μg/ml of penicillin G, whereas an *srsA* mutant (Δ*srsA*) cannot grow. Introduction of *srsA* on plasmid pKUM10-*srsA* restores the penicillin resistance to the parental level [26]. The *ars* operon in *A. vinelandii* is responsible for the biosynthesis of the phenolic lipids, the major lipids in the multilayered cyst coat. The long-chain fatty acids synthesized by type I fatty acid synthases, ArsA and ArsD, are converted to alkylresorcinols by ArsB and to alkylpyrones by ArsC. In the ArsB (and ArsC) reaction, the long-chain fatty acids produced by ArsA and ArsD remaind attached to the ACP domain of ArsA and were transferred hand-to-hand to the active-site Cys residue of ArsB, which then uses these fatty acids as starters and carries out two or three extensions with malonyl-CoA to yield the phenolic lipids. Electron micrographs of an ultrathin section of cysts of *A. vinelandii* OP (upper) and a cell of mutant *arsB*::*aphII* (lower) show the central body (CB) in both cells but impaired cyst coat (Ex, exine; In, intine) in the mutant [23]. ORAS in *N. crassa* is responsible for the biosynthesis of pentaketide alkylresorcylic acid, using stearoyl-CoA as a starter, condensing four molecules of malonyl-CoA to give a pentaketide intermediate. Aldol condensation and aromatization of the intermediate, which is still attached to the enzyme, are followed by hydrolysis for release of the product as a resorcylic acid. Note that the timing of the hydrolysis of the product is different from that for ArsB.

HPQ melanin. A vivid contrast in THN melanin biosynthesis between streptomycetes and fungi is that the THN synthesized by the action of a type III PKS is used directly for condensation in the former [17], whereas the THN synthesized by the action of type I PKSs is first reduced, and the resulting 1,8-dihydroxynaphthalene is then condensed in the latter [20, 21]. In various streptomycetes, on the other hand, the THN synthesized by RppA is monooxygenated by the action of MomA, belonging to the "cupin" superfamily, to form flaviolin, which is further modified to give secondary metabolites [22].

2-2. Type III PKSs Responsible for Biosynthesis of Phenolic Lipids

As described above, the variety of the plant polyketide structures produced by our combinatorial biosynthesis approach depends on the substrate specificity and mode of cyclization of various type III PKSs that we used as a member of artificially assembled biosynthetic pathways. We therefore searched the genome databases to find type III PKSs with different enzyme properties, in the hope that some type III PKSs could be used for production of useful compounds. Once type III PKSs with novel enzymatic properties were discovered, these could become useful members as one of the steps of the artificial biosynthetic pathways. Among the type III PKSs we identified, ArsB and ArsC encoded by an *arsABCD* operon in *Azotobacter vinelandii* turned out to be an alkylresorcinol synthase and an alkylpyrone synthase, respectively [23] (Fig. 1). Ars stands for *Azotobacter* resorcinol synthesis. The alkylresorcinols are essential for formation of cysts (resting cells surrounded by a protective coat that confers resistance to various chemical and physical stresses) because the lack of alkylresorcinol synthesis caused by an *ars* mutation results in the formation of severely impaired cysts [23], in agreement with the fact that the major chemical components of the cyst coat are alkylresorcinols [24]. Further *in vivo* and *in vitro* analysis of the Ars enzymes established their functions as follows [25]. ArsA and ArsD, both of which are type I fatty acid synthases, are responsible for the synthesis of $C_{22\sim26}$ fatty acids from malonyl-CoA. Interestingly, the $C_{22\sim26}$ fatty acids still remain attached to the acyl carrier protein (ACP) domain of ArsA, which are transferred hand-to-hand to the active-site cysteine residues of ArsB and ArsC. The type III PKSs, ArsB and ArsC, then use the fatty acids as starter substrates and carry out two or three extensions with malonyl-CoA to yield the phenolic lipids.

S. griseus has an operon including three enzyme genes, *srsABC* (*Streptomyces* resorcinol synthesis) (Fig. 1). *In vivo* and *in vitro* analysis of the Srs enzymes showed that SrsA is a type III PKS responsible for synthesis of phenolic lipids, alkylresorcinols and alkylpyrones, from acyl-CoAs of various chain lengths as a starter substrate, one molecule of methylmalonyl-CoA and two molecules of malonyl-CoA, SrsB is a methyltransferase acting on the phenolic lipids to yield alkylresorcinol methyl ethers, and SrsC is a hydroxylase acting on the alkylresorcinol methyl ethers [26]. SrsA and ArsA, both of which use long-chain fatty acid-CoA and synthesize phenolic lipids, can be used as members of artificially biosynthetic pathways for production of phenolic lipids and their modified compounds. Besides the potential of Srs enzymes for combinatorial biosynthesis, an interesting finding was that an *srsA* mutant, which produces no phenolic lipids, is highly sensitive to β-lactam antibiotics, such as penicillin G and cephalexin [26]. Together with the fact that the alkylresorcinols are fractionated mainly in the cell wall/membrane fraction, these observations suggest that the phenolic lipids, perhaps associated with the cytoplasmic membrane because of their amphiphilic property, affect the characteristic and rigidity of the cytoplasmic membrane/peptidoglycan. In fact, 5-alkylresorcinols are readily incorporated into phospholipid bilayers and biological membranes, thereby causing considerable changes in their structures and properties [27].

We also revealed that a single open reading frame, named ORAS (2′-oxoalkylresorcylic acid synthase), in the filamentous fungus *Neurospora crassa* catalyzes the synthesis of pentaketide alkylresorcylic acids [28] (Fig. 1). ORAS prefers stearoyl-CoA as a starter substrate and condensed four molecules of malonyl-CoA to give a pentaketide intermediate, which is still attached to the enzyme. After aldol condensation and aromatization of the intermediate, the product is released as a resorcylic acid by hydrolysis. The function of the phenolic lipids in this fungus is unknown, because disruption of the *ORAS* gene gives no detectable phenotypic changes.

2-3. Alkylresorcinols as Important, but Overlooked, Membrane Lipids in a Variety of Organisms?

The biological functions of the alkylresorcinols as essential membrane lipids for encystment in *A. vinelandii* [23] and as probable membrane lipids conferring penicillin resistance on *S. griseus* [26] suggest that these phenolic lipids play an important role as minor components in the biological membrane in various bacteria. In fact, a computer search in the databases predicts the existence of *srs*- and *ars*-like operons in a wide variety of Gram-positive and negative bacteria. Among the *srs*-like operons in bacteria, we have determined the catalytic properties of the operon in *Bacillus subtilis*, which directs the synthesis of pyrone-type

lipids (manuscript in preparation). *srs*-like genes are present not only in prokaryotes but also in eukaryotes, including ORAS in the filamentous fungi and at least three such genes in the rice plant *Oryza sativa* (our unpublished results). Alkylresorcinols and alkylpyrones are therefore important, but thus far overlooked, membrane lipids in bacteria, fungi, and plants.

2-4. Cinnamate/4-coumarate:CoA Ligase

As the first step in the phenylpropanoid pathway in plants, phenylalanine is deaminated to yield cinnamic acid (**1a**) by the action of phenylalanine ammonia-lyase (PAL) (Fig. 2). Cinnamic acid (**1a**) is hydroxylated by cinnamate-4-hydroxylase (C4H) to *p*-coumaric acid (**2a**), which is then activated to *p*-coumaroyl-CoA (**2b**) by the action of 4-coumarate:CoA ligase (4CL). Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with *p*-coumaroyl-CoA to yield naringenin chalcone (**2e**), the precursor for a large number of flavonoids [29]. Naringenin chalcone (**2e**) is converted to naringenin (**2g**) by chalcone isomerase (CHI) or nonenzymatically *in vitro* [10, 11]. One of the barriers to the production of flavonoids and their related compounds in microorganisms by means of assembling biosynthetic genes to form an artificial pathway is the difficulty in expression of active, membrane-bound C4H. This enzyme is not expressed efficiently in bacteria due to its instability and the lack of its cognate cytochrome P450 reductase in the host.

During our mining of the "treasure trove" in *Streptomyces* [15], we found cinnamate/4-coumarate:CoA ligase in *Streptomyces coelicolor* A3(2), named ScCCL (*S. coelicolor* A3(2) cinnamate/4-coumarate:CoA ligase), catalyzing the conversion of both cinnamic acid (**1a**) and *p*-coumaric acid (*p*-hydroxycinnamate) (**2a**) into the corresponding CoA thiol esters **1b** and **2b** at almost the same efficiency [14] (Fig. 3). The use of the *Streptomyces* ScCCL enzyme would bypass the C4H step for the production of pinocembrin chalcone (**1e**; Fig. 4) from phenylalanine *via* the phenylpropanoid pathway. Furthermore, when CHS and CHI in the artificial pathway use intermediates starting from phenylalanine and tyrosine, due to their generous substrate specificity we could successfully produce pinocembrin (**1g**) from phenylalanine and naringenin (**2g**) from tyrosine, as described below.

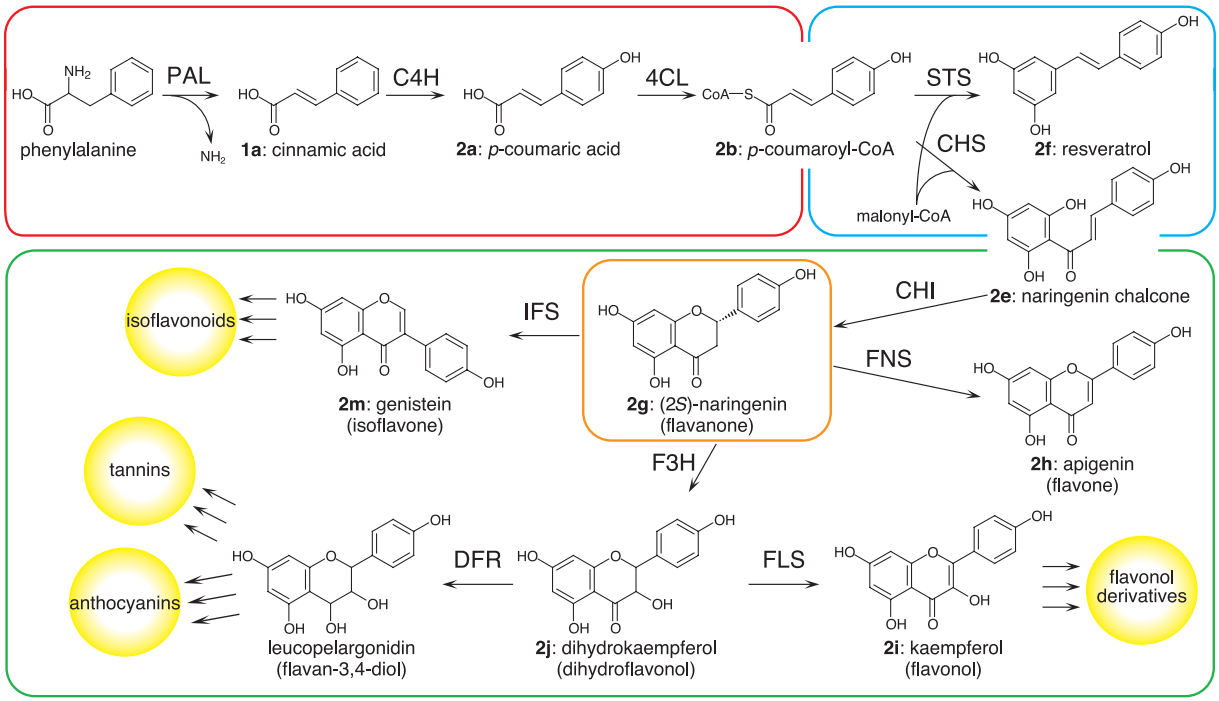

Fig. 2 The flavonoid biosynthetic pathway in plants.

The phenylpropanoid biosynthetic pathway leading to production of flavanones, the key intermediate in the flavonoid biosynthetic pathway in plants, and stilbenoids, which are then modified into a variety of plant-specific polyphenols. For the multiplasmid approach shown in Fig. 7, the pathway is divided into three: substrate synthesis (surrounded with a red line), polyketide synthesis (blue), and polyketide modification (green).

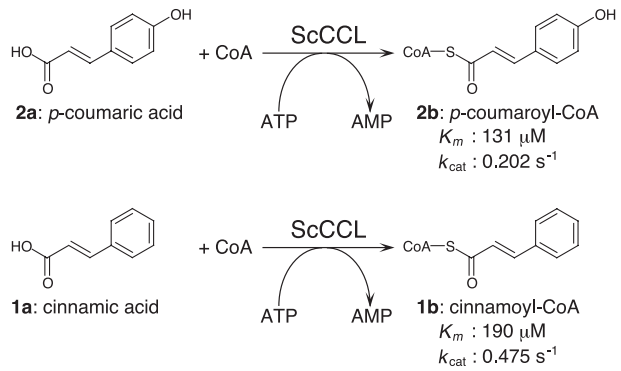

Fig. 3 Substrate specificity of cinnamate/4-coumarate: CoA ligase (ScCCL) from *S. coelicolor* A3(2).

3. Combinatorial Biosynthesis: Design and Construction of Artificial Biosynthetic Pathways for Fermentative Production of Flavanones and Stilbenes by *E. coli*

3-1. Production of (2*S*)-flavanones

Our strategy to produce flavanones, pinocembrin (**1g**) and naringenin (**2g**), by microorganisms was to design and express an artificial phenylpropanoid pathway (Fig. 4A). This was accomplished by assembling *PAL* from the yeast *Rhodotorula rubra*; *4CL* (*ScCCL*) from the actinomycete *S. coelicolor* A3(2); *CHS* from the licorice plant *Glycyrrhiza echinata*; and *CHI* from the plant *Pueraria lobata* on a single pET plasmid in *E. coli* (Fig. 4B) [5, 6, 30]. To maximize the expression of the four genes, various constructions of the artificial gene clusters were tested: for example, the arrangement of *PAL*, *4CL*, *CHS* and *CHI* such that the termination codon of the preceding gene overlapped with the start codon of the following gene. This type of gene organization, called translational coupling, is often found for sets of functionally related genes, such as for antibiotic biosynthesis and production of xenobiotics. The construction that proved to be optimal, however, employed an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible T7 promoter and a synthetic ribosome-binding sequence in front of each of the four genes (plasmid pET-P_{T7}-4GS) in a *recA*$^-$ host. Furthermore, the flavanone yields were greatly increased when the two subunit genes, *accBC* and *dtsR1*, of acetyl-CoA carboxylase from *Corynebacterium glutamicum* were expressed under the control of the T7 promoter and the synthetic ribosome-binding sequence in a pRSFDuet-derived plasmid (plasmid pRSF-ACC) different from the pET vector containing the synthetic pathway. We assume that enhanced expression of the acetyl-CoA carboxylase using a foreign promoter free of any regulation leads to a great increase in the intracellular pool of malonyl-CoA, which results in enhanced production of the flavanones. The concentration of malonyl-CoA, one of the precursors of flavanones (Fig. 2 and Fig. 4B), in *E. coli* cells is only 4∼90 μM (0.01∼0.23 nmol/mg dry weight) [31].

The medium and fermentation conditions for the recombinant *E. coli* cells to produce flavonoids in high yield were determined [6]. The recombinant *E. coli* cells harboring pET-P_{T7}-4GS and pRSF-ACC were first grown at 26°C in Luria-Bertani (LB) medium containing antibiotics to maintain the plasmids and IPTG to induce the T7 promoters, harvested, resuspended in minimal M9 medium to give a concentration of 50 g wet cells/liter, and incubated at 26°C for 36 to 60 hours. When concentrated cells were incubated in M9 medium, no cell growth but a constant consumption of glucose was observed. Under the incubation conditions, therefore, few metabolic contaminants derived from the metabolism of the *E. coli* host were accumulated in the supernatant, which facilitated easy purification of the flavanones from the supernatant. The yields of (2*S*)-pinocembrin (**1g**) from 3 mM phenylalanine exogenously added and (2*S*)-naringenin (**2g**) from 3 mM tyrosine were both 60 mg/liter. The optical purity of the naringenin produced by the *E. coli* cells was 70% enantiomeric excess (ee). The rather low ee is perhaps due to auto-racemization from the 2*S*- to 2*R*-form during purification, because (2*S*)-naringenin is readily racemized [32].

3-2. Production of Stilbenes

Stilbenes are polyketides exclusively produced by plants and have various biological functions, such as phytoalexins and antioxidants [33]. In addition, a variety of their bioactivities contributing to human health, including antioxidant activity, anti-inflammatory activity and anti-tumor activity have been shown. Resveratrol (**2f**; Fig. 5), a representative of stilbenes found in grapes and other food products, has attracted an increasing interest due to its inhibitory activity, both *in vitro* and *in vivo*, against three stages of carcinogenesis: initiation, promotion and progression [34∼36]. Therefore, several attempts to produce resveratrol by recombinant microorganisms were reported [37∼39]. However, these reports deal with bioconversion of intermediates, but not fermentative production starting with the amino acids.

Stilbenes are biosynthesized from tyrosine and phenylalanine (for part of the pathway, see Fig. 5), which are converted to the corresponding carboxylic acids, cinnamic acid and *p*-coumaric acid, by the activity of PAL. These acids are then activated to CoA esters **1a** and **2a** by 4CL, which become the substrates of stilbene synthase (STS), the key type III PKS in stilbene synthesis, and

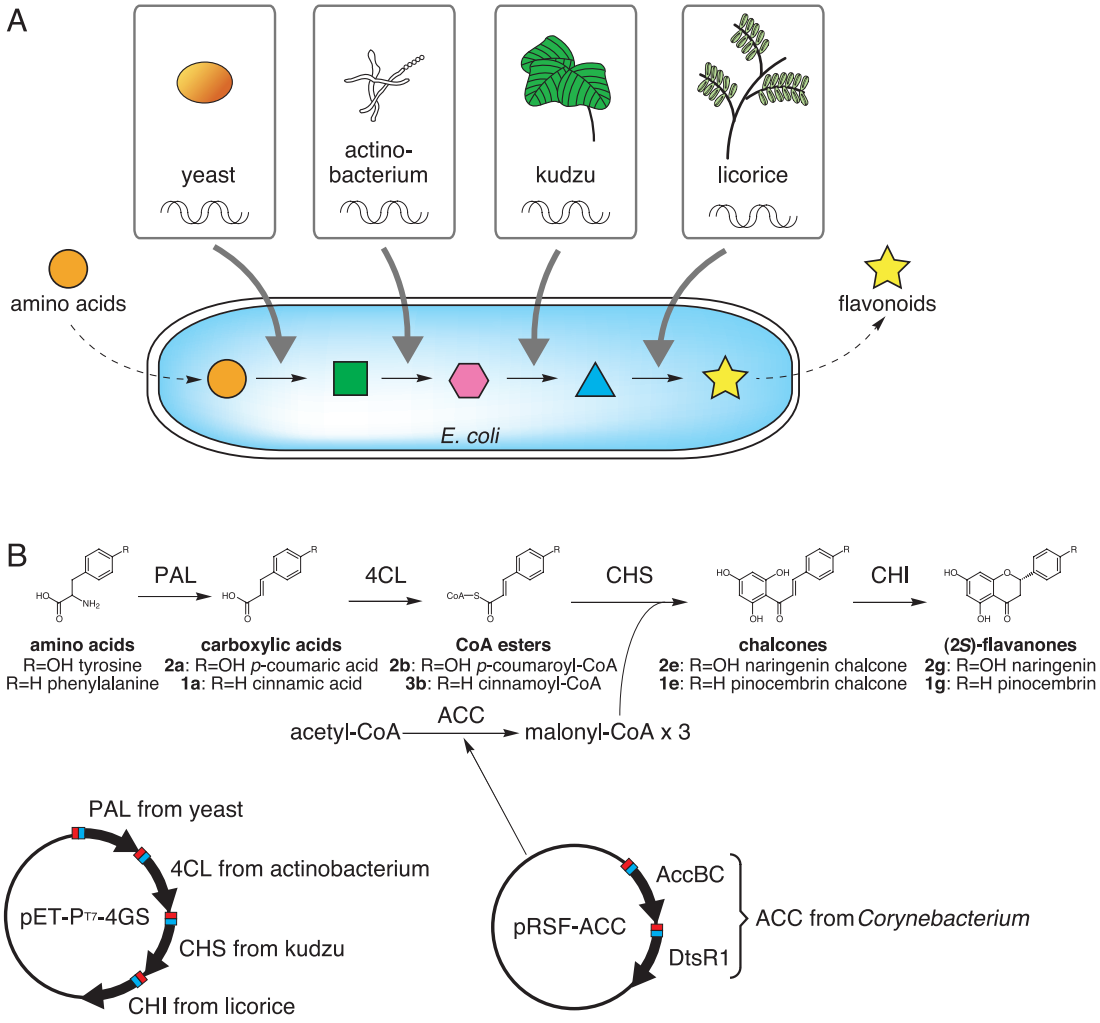

Fig. 4 The concept of combinatorial biosynthesis used for production of plant-specific polyketides by microorganisms (A) and a design of an artificial biosynthetic pathway for production of flavanones (B).

A. An artificial biosynthetic pathway, consisting of enzyme genes from yeast (*R. rubra*), actinomycete (*S. coelicolor*), kudzu plant (*P. lobata*), and licorice plant (*G. echinata*), is introduced and expressed in *E. coli*, where amino acids, tyrosine and phenylalanine, are successively converted to the corresponding flavanones and secreted in the medium. B. The biosynthetic pathway from the amino acids to the corresponding flavanones: naringenin (**2g**) from tyrosine and pinocembrin (**1g**) from phenylalanine. The *PAL*, *4CL* (*ScCCL*), *CHS*, and *CHI* genes are each under the control of the T7 promoter (shown in red) and a synthetic ribosome-fastening sequence (shown in blue) on a pET vector. pRSF-ACC, carrying the acetyl-CoA carboxylase genes, *accBC* and *dtsR1*, from *C. glutamicum*, is to increase the intracellular pool of malonyl-CoA.

condensed with three molecules of malonyl-CoA, resulting in stilbenes, resveratrol (**2f**) from tyrosine and pinosylvin (**1f**) from phenylalanine. For production of stilbenes in microorganisms, the only thing we had to do was just to replace the *CHS* gene in pET-P_{T7}-4GS for the flavanone biosynthetic pathway in Fig. 4B with an *STS* gene. This is one of the advantages in assembling a biosynthetic pathway for a certain product; replacing a single enzyme, the type III PKS, gives a different product, the structure of which depends on its catalytic properties, number of extensions, extender substrates employed, and mode of cyclization of the extended intermediate.

Because the PAL from *R. rubra*, 4CL from *Lithospermum erythrorhizon* (gromwell), and STS from *Arachis hypogaea* use both tyrosine- and phenylalanine-derived substrates, we designed a stilbene biosynthetic pathway in *E. coli* on two plasmids: pCDF-PAL/LE4CL-1 that contained the *PAL* and *4CL* genes on vector pCDFDuet-1 and pET-STS that contained the *STS* gene on vector pETDuet-1 [40, 41]. These three gene products successively convert tyrosine, for example, to *p*-coumaric acid by PAL, to *p*-coumaroyl-CoA (**2a**) by 4CL, and finally to resveratrol (**2f**) by STS.

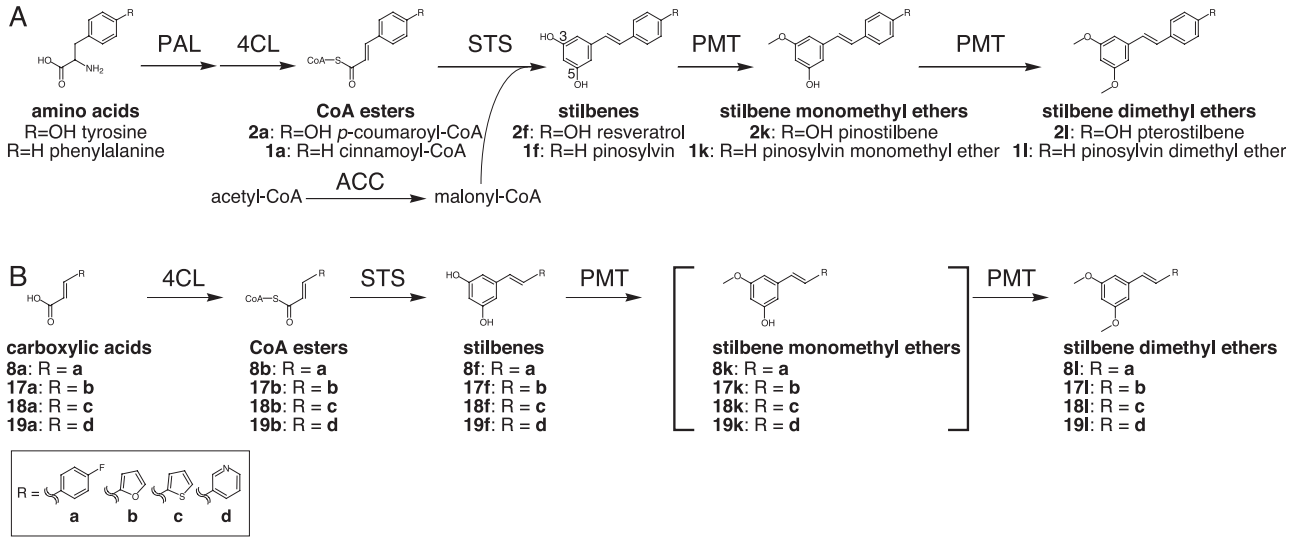

Fig. 5 Biosynthesis routes of stilbenes and stilbene methyl ethers constructed in recombinant *E. coli*.

A. By the sequential actions of PAL, 4CL and STS, tyrosine and phenylalanine are converted to stilbenes, resveratrol (**2f**) and pinosylvin (**1f**), respectively. Depending on the substrate specificity of OsPMT, the stilbenes are methylated at the 3- and 5-positions to yield the corresponding mono- (**2k** and **1k**) and di-methylated stilbenes (**2l** and **1l**). B. Unnatural stilbenes, produced by precursor-directed biosynthesis (see Figs. 7 and 8), are also methylated by OsPMT. The carboxylic acids supplemented as precursors were *p*-fluorocinnamic acid (**8a**), 3-(2-furyl)acrylic acid (**17a**), 3-(2-thienyl)acrylic acid (**18a**), and 3-(3-pyridyl)acrylic acid (**19a**). Dimethylated stilbenes (**8l**, **17l**, **18l** and **19l**) were detected as the products, but monomethylated stilbenes (**8k**, **17k**, **18k** and **19k**) were undetectable.

When the cells (25 g/liter wet weight) harboring pCDF-PAL/LE4CL-1, pET-STS and pRSF-ACC were incubated at 26°C for 60 hours in the presence of 3 mM tyrosine or phenylalanine, glucose, antibiotics to maintain the plasmids, and IPTG to induce the T7 promoter in M9 minimal medium, the yield of resveratrol (**2f**) or pinosylvin (**1f**) was 37 or 20 mg/liter, respectively.

4. Modifications of Flavanones and Stilbenes by Decorating Enzymes

4-1. Production of Flavones and Flavonols

In the biosynthesis of a variety of flavonoids (Fig. 2), (2*S*)-flavanones (**2g**) are key intermediates that are modified to a variety of compounds, such as anthocyanins and tannins, by modification enzymes. Hydroxylation of flavanones by flavanone 3β-hydroxylase (F3H) and flavonol synthase (FLS) results in flavonols. Flavone synthase (FNS) modifies flavanones into flavones. Because we established an *E. coli* system for production of flavanones, we could modify the flavanones produced by introducing the F3H/FLS and FNS genes into the recombinant *E. coli* cell. Cloning of F3H and FLS genes from the plant *Citrus* species under the control of the T7 promoter and the ribosome-binding sequence in pACYCDuet-1 and introduction of the plasmid in the recombinant *E. coli* host harboring pET-P_{T7}-4GS and pRSF-ACC led to production of flavonols: kaempferol (**2i**; 15.1 mg/liter) from 3 mM tyrosine supplemented and galangin (**1i**; 1.1 mg/liter) from 3 mM phenylalanine supplemented [42]. Similarly, introduction of an FNS gene from *Petroselinum crispum* on pACYCDuet-1 in the *E. coli* host led to production of flavones: apigenin (**2h**; 13 mg/liter) from tyrosine and chrysin (**1h**; 9.4 mg/liter) from phenylalanine.

Biochemistry of the enzymes to modify flavones and flavonols has been advanced [43, 44]. These include reduction, hydroxylation, sugar transfer, acylation, prenylation, and *O*-methylation. For example, prenylation, a critical step in the biosynthesis of many natural products, has been used to synthesize natural and unnatural chalcones showing more potent bioactivities [45, 46]. In addition, many bacterial enzymes have potentially activities to modify flavonoids. Prenyltransferases of bacterial origins, especially from the genus *Streptomyces*, are useful tools for prenylation of not only flavonoids but also other aromatic compounds [47, 48]. As an example for flavonoid modification by bacterial enzymes, we showed that an engineered biphenyl dioxygenase, shuffled from the enzymes from *Pseudomonas pseudoalcaligenes* and *Burkholderia cepacia*, modifies flavone to yield 2′,3′-dihydroxyflavone (a major product) and 3′-hydroxyflavone

(a minor product). It also modifies flavanone to yield 2′,3′-dihydroxyflavanone (a major product), 2′- and 3′-hydroxyflavanone (minor products) [49]. 2′, 3′-Dihydroxyflavanone was a novel compound. The modification enzymes of bacterial origins, as well as plant origins, can be readily incorporated as members in the artificial biosynthetic pathways, which should lead to construction of a larger library of natural and unnatural flavonoid and stilbenoid compounds.

4-2. Production of Stilbene Methyl Ethers

Despite a variety of bioactivities of stilbenes contributing to human health, they are rapidly absorbed and metabolized when given orally. In some cases, modification of the resveratrol scaffold by hydroxylation and methylation results in enhancement of its bioactivities. For instance, 3,5-*O*-methylated stilbenes [pterostilbene (**2l**; Fig. 5) and 3-hydroxypetrostilbene] are better apoptosis-inducing agents towards several tumor cells [50, 51] (Fig. 5). In addition to chemopreventive activity, methylated resveratrols, such as desoxyrhapontigenin (4′-*O*-methyl-resveratrol) and pinostilbene (**1k**: 3-*O*-methyl-resveratrol), exhibit higher CYP1A1 inhibitory activity than resveratrol (**2f**) [52]. Furthermore, a pinosylvin monomethyl ether (**1k**) as a nematicidal agent from *Pinus massoniana* shows a ten-times smaller LC_{50} value than pinosylvin (**1f**) [53]. These observations show usefulness of methylated stilbenes as bioactive substances.

Since we had developed a system for high-yield production of resveratrol (**2f**) from tyrosine and pinosylvin (**1f**) from phenylalanine, it was fairly easy for us to produce methylated stilbenes. We first searched for a possible pinosylvin methyltransferase (PMT) *in silico* and found a candidate, Os08g06100, in the genome of the rice *O. sativa*. The cDNA of the candidate gene (*OsPMT*) was cloned from the rice cell and expressed in *E. coli*, and its methytransferase activity toward pinosylvin (**1f**) was confirmed [41]. The *OsPMT* cDNA was then placed under the control of the T7 promoter and a synthetic ribosome-binding sequence in pACYCDuet-1 vector (plasmid pACYC-OsPMT) and introduced in *E. coli* carrying the *PAL* and *4CL* genes on pRSF-PAL/LE4CL-1, the *STS* gene on pET-STS, and the *ACC* gene on pRSF-ACC, thus creating a pathway consisting of PAL, 4CL, STS, ACC, and OsPMT on four different, compatible plasmids in *E. coli* [41] (Fig. 5).

Supplementation of 3 mM tyrosine to the recombinant *E. coli* cells resulted in production of 18 mg/liter pinostilbene (resveratrol monomethyl ester; **2k**) as a major product and 6 mg/liter pterostilbene (3,5-dimethyl resveratrol; **2l**) as a minor product [41]. Therefore, OsPMT methylates the hydroxy groups at positions 3 and 5 of the stilbene scaffold. This system is therefore superior in the selectivity of methylation to random methylation of hydroxyl groups by chemical modification. Similarly, supplementation of 3 mM phenylalanine resulted in production of pinosylvin monomethyl ether (**1k**) and pinosylvin dimethyl ether (**1l**) almost in the same yield of 27 mg/liter. These results suggested that OsPMT prefers pinosylvin (**1f**) to resveratrol (**2f**) as a substrate. The relaxed substrate specificity of OsPMT thus turned out to be useful for methylating unnatural stilbenes at their 3- and 5-positions, as described below.

5. One-pot Synthesis of Isoflavone by Coincubation of Genetically Engineered *E. coli* and *Saccharomyces cerevisiae* Cells

Isoflavonoids are synthesized predominantly in soybeans and other leguminous plants where they act as antimicrobial phytoalexins, signal molecules of nodulation genes of symbiotic microorganisms, stimulators of fungal spore germination, insect antifeedants, and allelochemicals [54]. In addition, isoflavonoids have several beneficial effects on human health, such as chemopreventive actions against cancer, osteoporosis, and cardiovascular disease [54]. Genistein (**2m**; Fig. 2), which is a common precursor of the biosynthesis of isoflavonoids, has attracted much attention because of its phytoestrogen activity [54]. Several groups have tried to produce genistein in nonlegume plants but the yields were very low probably because of competitive use of naringenin (**2g**) between isoflavone synthase (IFS) and the endogenous flavonoid pathway [44, 55, 56]. On the other hand, microbial production of isoflavonoids has been hampered because IFS, a key enzyme catalyzing the aryl migration of (*S*)-naringenin (**2g**) to yield isoflavonoid (**2m**) [57∼59], is a membrane-bound cytochrome P450 enzyme that requires a specific electron transfer system. It is known that the aglycons of isoflavonoids are directly absorbed from the stomach when orally admitted [60], although they are produced as β-glycosides by plants. An efficient system for fermentative production of isoflavonoids has therefore been awaited.

In order to employ the yeast *Saccharomyces cerevisiae* as the host for conversion of (2*S*)-naringenin (**2g**; Fig. 6) to genistein (**2m**), we first confirmed that IFS from *G. echinata* was functionally expressed in *S. cerevisiae* and that the host converted exogenously added naringenin to genistein, as was observed by Akashi *et al.* [57]. Our approach was to produce genistein (**2m**) from tyrosine by separating the biosynthetic route into two; one was for

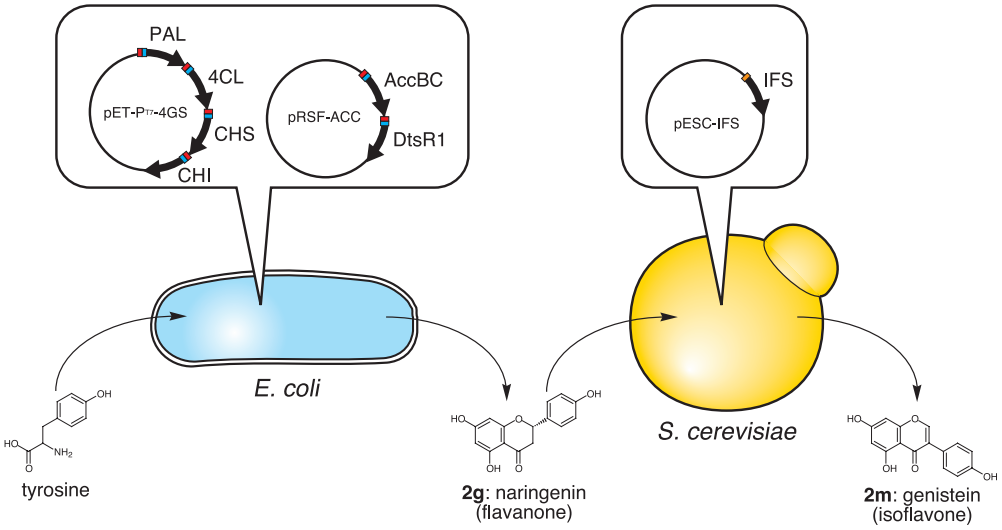

Fig. 6 One-pot synthesis of genistein by coincubation of recombinant *E. coli* harboring pET-P$_{T7}$-4GS and pRSF-ACC and *S. cerevisiae* carrying pESC-IFS in potassium phosphate buffer.

The T7 promoter (shown in red) and a ribosome-binding sequence (in blue) in front of the genes in *E. coli* are indicated. The galactose-inducible *GAL* promoter (in yellow) in front of the *IFS* gene in yeast is also indicated. The naringenin (**2g**) produced in and excreted from the *E. coli* cell is incorporated in the yeast cell, converted to genistein (**2m**), and excreted in the medium.

production of (*S*)-naringenin (**2g**) from tyrosine by using the *E. coli* system and the other was for conversion of the (*S*)-naringenin (**2g**) into genistein (**2m**) by using a yeast system [61]. Thus, our approach for "one-pot production" of genistein (**2m**) was coincubation of the *E. coli* cells, producing about 60 mg/liter (*S*)-naringenin from tyrosine, and the yeast cells, carrying the *IFS* gene from *G. echinata* under the control of the galactose-inducible *GAL* promoter in pESC vector (Fig. 6). Because of the permeability of naringenin through the *E. coli* and yeast membranes, coincubation of the recombinant *E. coli* expressing the (*S*)-naringenin biosynthetic enzymes and the recombinant *S. cerevisiae* cells expressing IFS would lead to the conversion of the (*S*)-naringenin (**2g**) produced by and secreted from the *E. coli* cells into genistein (**2m**) by the *S. cerevisiae* cells as an IFS catalyst. As expected, simultaneous incubation of 0.5 g each (wet weight) of the *E. coli* and yeast cells at 26°C for 60 hours in 20 ml potassium phosphate buffer containing 3 mM tyrosine as the starter, 4% galactose for induction of the *GAL* promoter in front of the *IFS* gene, 1 mM IPTG for induction of the T7 promoter in front of the (*S*)-naringenin biosynthetic genes, and antibiotics for maintenance of the plasmids gave about 6 mg/liter genistein. As shown in Fig. 6, supplementation of tyrosine to a flask containing two different enzyme bags in potassium phosphate buffer resulted in the production of an isoflavone (**2m**). In this system, a considerable amount of a mixture of (*RS*)-naringenin still remained in the broth, suggesting that not all the (*S*)-isomer molecules were converted into genistein by IFS. Improvement of the coincubation conditions enhanced the isoflavonoid yield up to 100 mg/liter (unpublished results).

An advantage of flavonoid production in yeasts or fungi is their ability to express functionally active microsomal cytochrome P450 enzymes, which are usually difficult to be expressed in an active form in bacterial cells. There are various microsomal cytochrome P450 enzymes that are involved in the flavonoid biosynthesis pathway [62]. Therefore, this approach, combining bacterial cells and eukaryotic cells in a pot, should enable us to generate a wider library of natural and unnatural products than any of the previously reported systems. Our system for genistein production by multiple microorganisms makes a model system for production of not only plant-specific polyketides but also a wider variety of other compound classes in future.

6. Precursor-directed Biosynthesis of Unnatural Flavonoids and Stilbenoids

6-1. Production of Unnatural Flavonoids

Once flavonoids, stilbenes and their related compounds are produced by microorganisms carrying an artificial biosynthetic pathway, it is not difficult to modify or

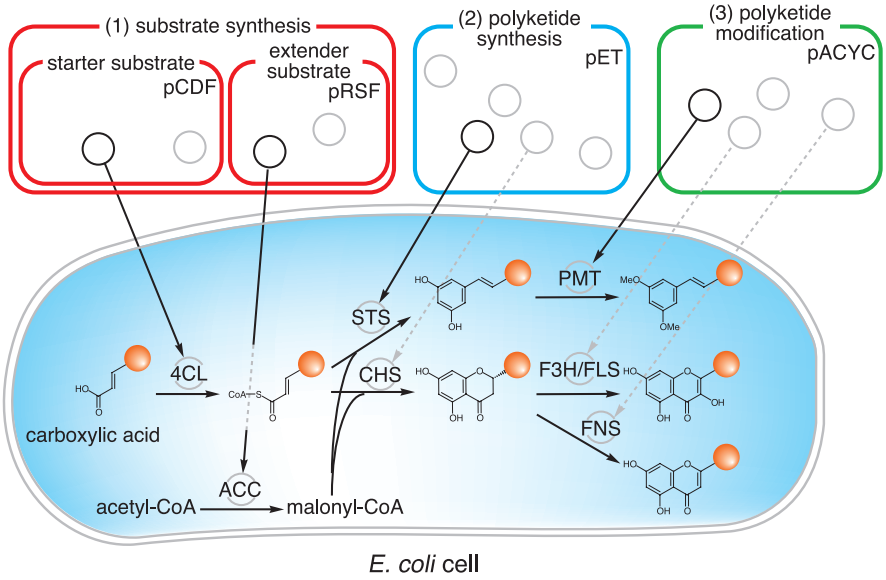

Fig. 7 Precursor-directed biosynthesis of unnatural flavonoids and stilbenoids.

The carboxylic acids supplemented as precursors to the system are shown schematically. The artificial pathways are divided into three steps (see Fig. 2): substrate synthesis, polyketide synthesis, and polyketide modification. The three steps are each on a plasmid. The actual precursors supplemented and the resulting products are summarized in Fig. 8.

decorate the products by further adding some decorating enzyme genes to the pathway, as already described for the production of flavonols, flavones and methylated stilbenes. We therefore expect the production of novel compounds when some bacterial decorating enzymes, not present in plants, are used. In addition, it is also possible to produce unnatural compounds by precursor-directed biosynthesis (Fig. 7). This technique was successfully applied to production of novel polyketides by a type I PKS mutant that was blocked in the early stage of the biosynthesis of a natural product [63]. Polyketide synthesis by the mutant enzyme was restored by the introduction of natural precursors that were incorporated into the biosynthetic scheme at a point after the block caused by the mutation. Introduction of analogs of natural precursors, in turn, gave novel compounds. We divided the biosynthesis pathway of plant polyketides into three: substrate synthesis, polyketide synthesis, and postpolyketide modification. The genes involved in these three steps were each placed on a plasmid with different replication origins and selectable markers and simultaneously introduced in *E. coli* (Fig. 7), as in the above-described cases of the production of flavanones and their related compounds. One of the advantages of this multiplasmid approach [64] is that construction of recombinant biosynthetic pathways by cotransformation of plasmids is rapid and efficient, avoiding the laborious and time-consuming tasks required to construct plasmids for individual biosynthetic steps. For the substrate synthesis from carboxylic acids to their corresponding CoA compounds, *4CL* on pCDF vector was used. In addition, pRSF-ACC was also used to increase the intracellular pool of malonyl-CoA as an extender substrate. For the polyketide synthesis from the CoA compounds, type III PKSs, such as *CHS* for production of flavanones and *STS* for production of stilbenes, were used. As described later, the use of a curcuminoid synthase (*CUS*) gene as a type III PKS at this step led to efficient production of curcuminoids. For polyketide modification, *F3H*/*FLS* for flavonol production, *FNS* for flavone production, and *OsPMT* for production of methylated stilbenes were successfully used.

For production of unnatural flavanones by the system, unnatural carboxylic acids, such as fluorocinnamic acids (**8a**~**11a**), furyl (**15a** and **17a**), thienyl (**16a** and **18a**), and naphthyl (**20a**) acrylic acids, gave the corresponding unnatural flavanones (**8g**~**11g**, **15g**~**18g**, and **20g**) [40] (Fig. 8). Of the unnatural flavanones produced, novel compounds that have not been reported in the literature are shown in blue in Fig. 8. The yields of the unnatural flavanones derived from **8a**, **15a** and **16a** reached ca. 50 to 100 mg/liter, which is comparable to those (70 to 90 mg/liter) of the natural flavanones derived from **1a** and **2a**. In contrast, the yields of natural (**1c**) and unnatural (**16c**) pyrones were about 3 mg/liter, in agreement with the property of the CHS that it produces byproduct pyrones in small amounts. The unnatural flavanones were further

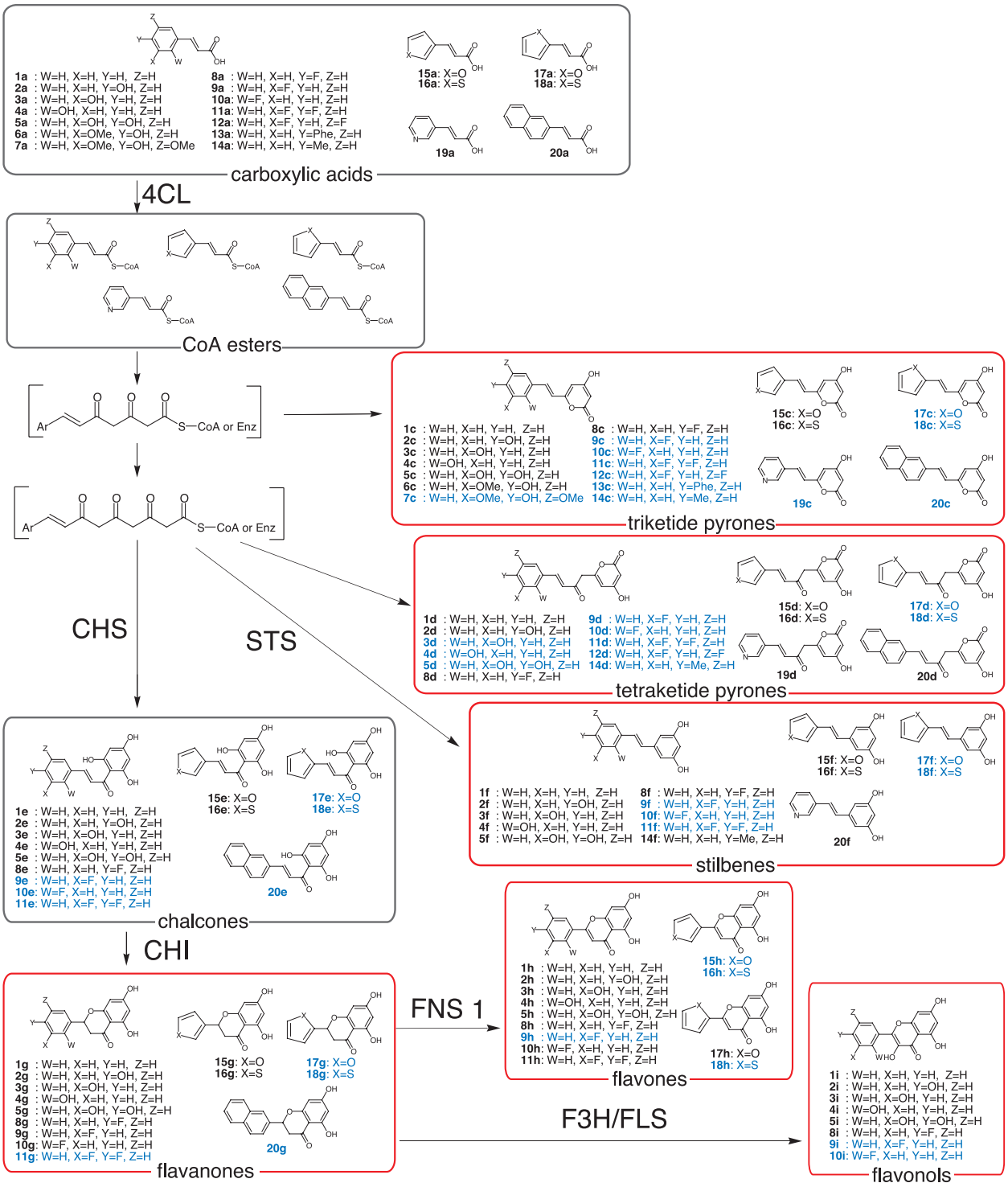

Fig. 8 Plant polyketides produced by recombinant *E. coli* carrying artificial biosynthetic pathways by means of precursor-directed biosynthesis.

The polyketides synthesized are surrounded by red squares. Novel compounds, which had not then been reported in the literature, are indicated in blue. Reproduced from Katsuyama *et al.* [40].

modified by FNS and F3H/FLS, resulting in the production of 30 to 50 mg/liter unnatural flavones derived from **8a**, **15a**, and **16a**. These findings suggest that the FNS modifies a broad spectrum of unnatural flavanones. However, the unnatural flavonols derived from **8a**, **15a**, and **16a** were produced only in trace amounts, and considerable amounts of the flavanones remained intact, which suggests no efficient conversion of the flavanones to flavonols by F3H/FLS. These data showed that the system, in which the multiplasmid approach and precursor-directed biosynthesis are combined, is useful and efficient for production of novel polyketides.

6-2. Production of Unnatural Stilbenoids

Replacement of the CHS with the STS for the polyketide synthesis step led to production of 15 stilbenes (**1f**∼**5f**, **8f**∼**11f**, and **14f**∼**19f**) (Fig. 8). The yields of stilbenes derived from the unnatural carboxylic acids were 55 to 130 mg/liter, which was comparable to those of 130 to 170 mg/liter, derived from the natural carboxylic acid, **1a** and **2a**. Besides stilbenes, negligible amounts of triketide pyrones (**1c**∼**20c**) and tetraketide pyrones (**1d**∼**5d**, **8d**∼**12d**, and **14d**∼**20d**) were also observed, showing the occurrence of derailment reactions by the STS.

We also tested whether OsPMT acts on unnatural stilbenes and produces methylated stilbene derivatives (Fig. 5B). As described above, STS cyclized the intermediates derived from *p*-fluorocinnamic acid (**9a**), 3-(2-furyl)acrylic acid (**17a**), 3-(2-thienyl)acrylic acid (**18a**), and 3-(3-pyridyl)acrylic acid (**19a**) to yield the corresponding stilbenes. Incubation of the recombinant *E. coli* harboring pCDE-LE4CL-1, pET-STS, and pACYC-OsPMT in the presence of these carboxylic acids gave stilbene 3,5-dimethyl ethers (**9l**, **17l**, **18l**, and **19l**). Rough estimation showed that the yields of two unnatural stilbene ethers (**9l** and **18l**) were comparable to those of the natural stilbene dimethyl ethers produced from *p*-coumaric acid and cinnamic acid. However, the yields of **17l** and **19l** were lower. The promiscuous substrate specificity of OsPMT is promising when it is used as a member of gene clusters designed for combinatorial biosynthesis.

6-3. Bioactivity of Unnatural Flavonoids and Stilbenes

Do the unnatural plant polyketides produced by this system exhibit some bioactivity? CYP1B1, which catalyzes hydroxylation of 17β-estradiol, is a major enzyme for carcinogenic estrogen metabolism and is thus involved in the metabolic activation of procarcinogens [65]. The ethoxyresorufin-*O*-deethylase (EROD) activity is commonly used to test the activity of CYP1B1. Preliminary examination of the inhibitory activity of ethyl acetate-extracts of the *E. coli* cells producing polyketides against the EROD activity of CYP1B1 showed that most of the polyketides had an inhibitory activity [40]. We then chose and purified five stilbenes (**1f**, **2f**, **8f**, **15f**, and **16f**) as representatives to determine their inhibition kinetics. The unnatural stilbenes (**8f**, **15f**, and **16f**) had distinct CYP1B1 inhibition kinetics similar to those of the natural stilbenes (**1f** and **2f**). Although among the unnatural polyketides tested there were no compounds that were superior in the CYP1B1 inhibition to the natural polyketides, these findings show that a library of unnatural products would be promising as a source for screening of bioactive compounds of various functions.

7. Combinatorial Biosynthesis of Curcuminoids in *E. coli*

7-1. Curcuminoid Synthase from the Rice *O. sativa*

Curcumin (**6n**; Fig. 9), a hydrophobic polyphenol derived from the rhizome of the herb *Curcuma longa* possesses diverse pharmacologic effects including anti-inflammatory, antioxidant, antiproliferative and antiangiogenic activities [66∼68]. In addition to its wide variety of biological and pharmacological activities, curcumin, the natural pigment that gives the spice turmeric its yellow color, has long been used as a traditional Asian medicine and a food additive. Curcumin (**6n**), bisdemethoxycurcumin (**2n**) and dicinnamoylmethane (**1n**) are chemically bis-α,β-unsaturated β-diketones and called curcuminoids. The intriguing structure of curcuminoids raised the question of whether a single type III PKS catalyzes all the steps in curcuminoid synthesis or only some of them, such as β-keto acid synthesis. Ten years had passed since Schröder [11] predicted that a type III PKS might be involved in curcuminoid synthesis until we recently discovered and characterized a curcuminoid synthase from the rice *O. sativa* [69].

During our screening for type III PKSs showing novel and interesting catalytic properties in various organisms, we came across a type III PKS, os07g17010, from *O. sativa*, which catalyzes the synthesis of curcuminoids from *p*-coumaroyl-CoA as a starter and malonyl-CoA as an extender [69] (Fig. 9A). The reaction by this enzyme, named CUS (curcuminoid synthase), begins with the thioesterification of the thiol moiety of Cys-174 by the starter molecule, *p*-coumaroyl-CoA (**2b**). Decarboxylative condensation of the first extender substrate, malonyl-CoA, onto the thioester of *p*-coumarate results in the formation of a diketide-CoA intermediate. Subsequent hydrolysis of the intermediate yields a β-keto acid, which is then joined to

the Cys-174-bound *p*-coumarate by decarboxylative condensation to form bisdemethoxycurcumin (**2n**). The incorporation of an additional phenylpropanoid unit violates the traditional head-to-tail model of polyketide assembly, *i.e.*, a switchover of the role of a diketide intermediate from a growing chain to an extender unit; the growing diketide intermediate is hydrolyzed to a β-keto acid that subsequently serves as the second extender to form curcuminoids. CUS also accepts other phenylpropanoid-derived CoA esters, such as cinnamoyl-CoA (**1b**) and feruloyl-CoA (**6b**), as a substrate to produce dicinnamoylmethane (**1n**) and curcumin (**6n**), respectively.

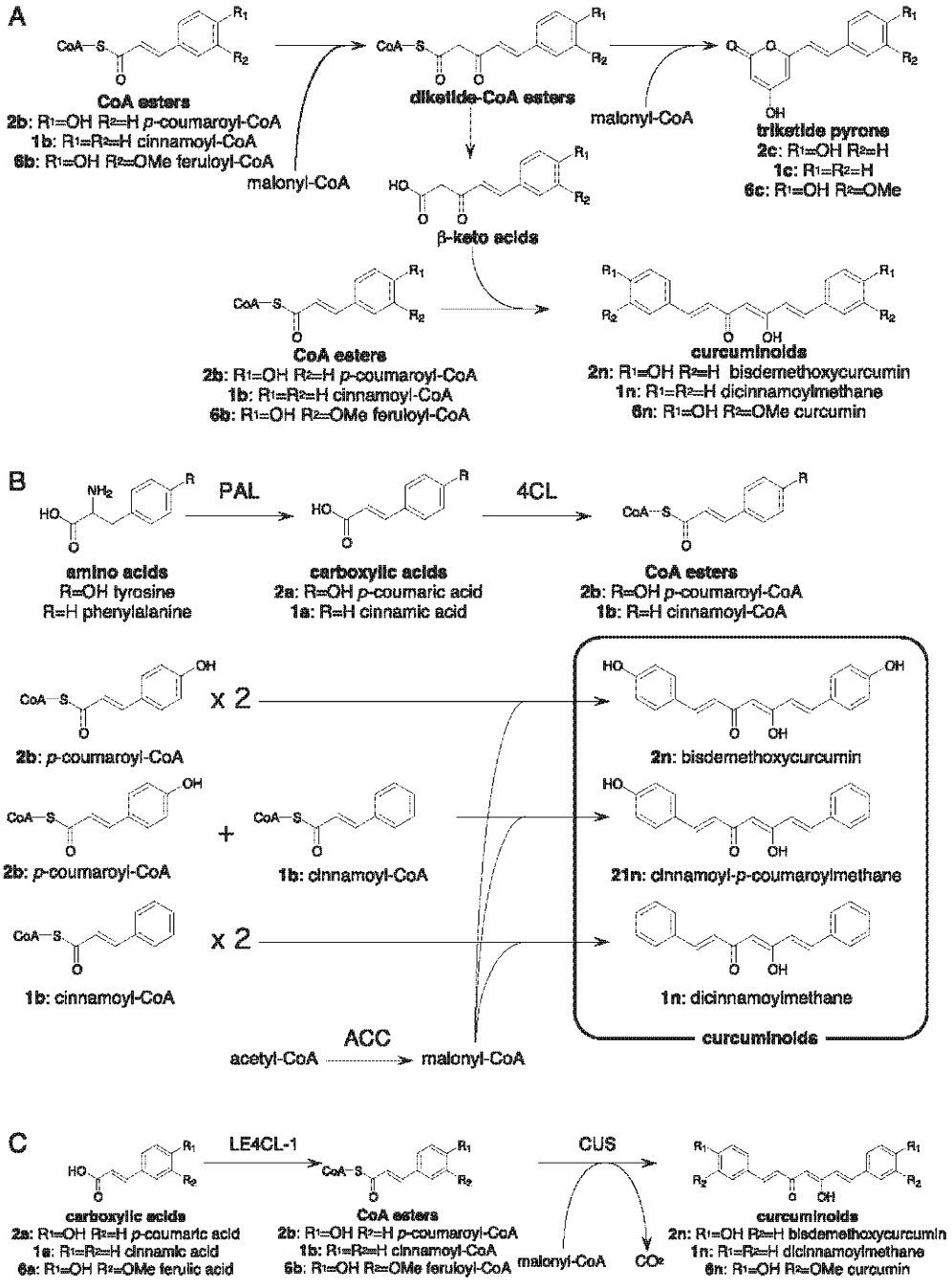

Fig. 9 Fermentative production of curcuminoids by *E. coli* carrying an artificial curcuminoid biosynthetic pathway.

A. Curcuminoid synthesis by CUS. See text for details. B and C. The artificial pathways for production of curcuminoids by *E. coli*. CUS converts the CoA esters, *p*-coumaroyl-CoA (**2b**) and cinnamoyl-CoA (**1b**), into curcuminoids (**2n**, **21n**, and **1n**). When two different CoA esters are present, CUS produces asymmetric curcuminoids in addition to symmetric curcuminoids. For instance, CUS yields cinnamoyl-*p*-coumaroylmethane (**21n**) from *p*-coumaroyl-CoA (**2b**) and cinnamoyl-CoA (**1b**).

The discovery of CUS prompted us to employ it as a type III PKS at the polyketide synthesis step in the artificial biosynthesis pathway for microbial production of plant-specific curcuminoids. Although curcuminoid synthesis in the rhizome of *C. longa* has not yet been elucidated, our preliminary study suggests involvement of another type III PKS, in addition to a CUS-type PKS (unpublished observation).

7-2. Production of Curcuminoids by *E. coli*

Our strategy to produce curcuminoids by *E. coli* on the basis of the catalytic properties of CUS is depicted in Fig. 9B [70]. This pathway included two steps: a substrate synthesis step for CoA esters synthesis from tyrosine and phenylalanine and a polyketide synthesis step for conversion of the CoA esters into curcuminoids by CUS. Plasmid pCDF-PAL/LE4CL-1 carrying both *PAL* and *4CL* was for the first step and plasmid pET-CUS carrying *CUS* under the control of the T7 promoter and a synthetic ribosome-binding sequence in pET16b vector for the second step. CUS was expected to condense two molecules of the CoA ester of the phenylpropanoid acid with one molecule of malonyl-CoA to produce curcuminoids.

Recombinant *E. coli* cells harboring pCDF-PAL/LE4CL-1, pET-CUS and pRSF-ACC were suspended to give a concentration of 25 g wet weight cells per liter in M9 minimal medium supplemented with 3 mM each of tyrosine (543 mg/liter) or phenylalanine (495 mg/liter), or both, glucose, antibiotics, and IPTG and incubated at 26°C for 60 hours. Under the incubation conditions, no increase of the cell mass was observed, which was advantageous in purifying the products from the M9 medium containing considerably small amounts of contaminants and metabolites of the host cell. In addition, the cytotoxicity of the curcuminoids, if any, caused no detectable effects on the *E. coli* cells. In this process, the *E. coli* cell could be considered a bag containing a whole set of enzymes for the synthesis of curcuminoids. Production of curcuminoids by the *E. coli* cells was apparent by a yellow color of the culture supernatant, when grown in liquid medium, and of the colony, when grown on solid medium (Fig. 10). Supplementation of tyrosine and phenylalanine greatly enhanced the production of bisdemethoxycurcumin (**2n**) up to 55 mg/liter and dicinnamoylmethane (**1n**) up to 110 mg/liter, respectively. When both phenylalanine and tyrosine were supplied, the predominant product was dicinnamoylmethane (**1n**). Furthermore, the yield of dicinnamoylmethane (**1n**) from phenylalanine was greater than that of bisdemethoxycurcumin (**2n**) from tyrosine. Although CUS prefers *p*-coumaroyl-CoA approximately twice as much as cinnamoyl-CoA as a substrate [69], the yield of dicinnamoylmethane (**1n**) was greater than that of bisdemethoxycurcumin (**2n**) in all reactions. This may be caused by the different rates of incorporation of tyrosine and phenylalanine into the pathway, which results from the substrate preferences of PAL and 4CL.

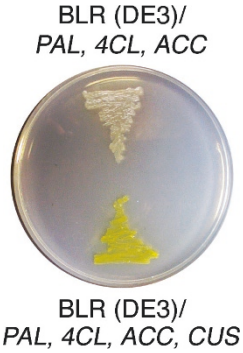

Fig. 10 Fermentative production of curcuminoids by *E. coli* that has been endowed with the ability of secondary metabolism.

E. coli BLR (DE3) carrying *PAL* and *4CL* on pCDF-PAL/LE4CL-1, *CUS* on pET-CUS, and *ACC* on pRSF-ACC was grown on routinely used LB agar without supplementation of amino acids. Curcuminoid production was apparent from a yellow color of the *E. coli* cells. Curcuminoids were not efficiently excreted into the medium. *E. coli* harboring pCDF-PAL/LE4CL-1 and pRSF-ACC was a negative control.

The yields of curcuminoids were improved by directly supplying phenylpropanoid acids to the *E. coli* cell carrying *4CL*, *CUS*, and *ACC*, on the assumption that the removal of the PAL step converting the amino acids to the corresponding carboxylic acids would increase the *p*-coumaroyl-CoA concentration in the *E. coli* cell (Fig. 9C). The recombinant *E. coli* cells were incubated at 26°C for 60 hours in the presence of 1 mM each of the phenylpropanoid acids (*p*-coumaric acid, cinnamic acid or ferulic acid), glucose, antibiotics, and IPTG in M9 minimal medium. When *p*-coumaric acid (**2a**), cinnamic acid (**1a**) or ferulic acid (**6a**) was supplied, the yield of bisdemethoxycurcumin (**2n**), dicinnamoylmethane (**1n**) or curcumin (**6n**), respectively, was about 100 mg/liter. In this reaction, the ratio of bisdemethoxycurcumin (**2n**) to triketide pyrone (**2c**) was improved 200-fold over the reaction starting from tyrosine, probably due to an increase of the *p*-coumaroyl-CoA concentration in the *E. coli* host.

The recombinant *E. coli* carrying *PAL*, *4CL*, *CUS*, and *ACC* produced an asymmetric curcuminoid, cinnamoyl-*p*-coumaroylmethane (**21n**), derived from cinnamic acid and *p*-coumaric acid, consistent with the *in vitro* study of CUS; it synthesizes asymmetric curcuminoids from two starter substrates [69]. Supplementation of both

p-coumaric acid and cinnamic acid to *E. coli* led to production of cinnamoyl-*p*-coumaroylmethane (**21n**). Similarly, demethoxycurcumin was produced from *p*-coumaric acid plus cinnamic acid, and cinnamoylferuloylmethane was produced from cinnamic acid plus ferulic acid. Approximately 7 mg/liter of cinnamoyl-*p*-coumaroylmethane (**21n**) was produced from an equal amount of cinnamic acid (1 mM or 148 mg/liter) plus *p*-coumaric acid (1 mM or 164 mg/liter), but with simultaneous production of 90 mg/liter of bisdemethoxycurcumin (**2n**). The production of the symmetric curcuminoid was perhaps due to the preference of *p*-coumaric acid to cinnamic acid as a substrate of CUS. The yield of cinnamoyl-*p*-coumaroylmethane (**21n**) might be improved by changing the substrate ratio of cinnamic acid to *p*-coumaric acid. A better understanding and engineering of CUS will much improve the yield of curcuminoids and the production ratio of a specific, desired compound. For the purpose of engineering CUS, we are currently trying to solve its three-dimensional structure. Furthermore, various carboxylic acids, including unnatural compounds, would be incorporated into the curcuminoid skeleton if the enzymes use them as a substrate, as we successfully synthesized natural and unnatural stilbenes by precursor-directed biosynthesis [40, 41]. Another point is that a type III PKS with novel enzymatic properties, such as CUS, can readily be incorporated as a member of the artificial biosynthetic pathway. Benzalacetone synthase (BAS) [71] and 2-pyrone synthase (2-PS) [72] are such examples.

7-3. Production of Curcumin from Rice Bran Pitch

Rice bran pitch is a dark and viscous oil, which is discharged in the course of production of rice edible oil from rice bran. Ten million tons of brown rice is produced every year and 10% of its weight is polished away before eating. This means that one million tons per year of the rice bran is lost. We planned to make use of the ferulic acid in the sheer waste as a substrate for the production of curcumin (**6n**). Ferulic acid (**6a**; Fig. 9C) was extracted by hydrolysis of γ-oryzanol by alkali, essentially according to Taniguchi *et al.* [73]. About 11 mg of ferulic acid (**6a**) was extracted from 500 mg of rice bran pitch.

E. coli (25 g wet weight/liter) harboring pCDF-LE4CL-1, pRSF-ACC and pET-CUS and 25 g/liter of $CaCO_3$ (to maintain a slightly alkaline pH) were added to the rice bran pitch medium, containing appropriate antibiotics and IPTG, and incubated at 26°C for 60 hours. In this reaction, about 60 mg curcumin (**6n**) was produced from 1 g of rice bran pitch. Previous studies reported that rice bran contains an array of bioactive compounds, such as oryzanols, phytic acid, ferulic acid, inositol hecaphosphate, and so on [74]. Zheng *et al.* [75] reported microbial production of vanillin from ferulic acid, which is contained in the waste residue of rice bran oil, by using *Aspergillus niger* and *Phycnoporus cinnabarinus*. Our present study also matches the trend of public requirements to make use of industrial wastes for the production of useful and valuable substances.

8. Concluding Remarks

Because of increasing interest in beneficial effects of flavonoids and stilbenoids, metabolic engineering in plants has been carried out for the purposes of increasing the yield of a specific compound by modulating some transcriptional factors and introducing foreign genes to increase levels of a rate-limiting biosynthetic enzyme and of modifying a compound by introducing a foreign gene. These efforts were summarized by Julsing *et al.* [3] and Ververidis *et al.* [4]. On the other hand, microorganisms have been used as a host only to check the enzyme properties of certain gene products. Microbiologists have much experience in metabolic engineering to increase the yields of some secondary metabolites including antibiotics and may have thought that similar strategies can be applied to flavonoids and stilbenoids once their biosynthetic pathways are constructed in microorganisms. Our success in fermentative production of flavonoids, stilbenoids and curcuminoids from the primary metabolites, phenylalanine and tyrosine, by *E. coli* by means of expressing artificially designed biosynthetic pathways makes this concept feasible. Examples are production of naringenin, eriodictyol, kaempferol and a few other compounds by *p*-coumaric acid-supplemented *E. coli* [76] and production of a raspberry ketone by *E. coli* [8]. This concept can be applied to production of many other compound classes by similar techniques, hopefully to paclitaxel and artemisnin as terpenoids and morphine, vincristine, and vinblastine as alkaloids.

In addition to rapid growth of microorganisms in natural and synthetic media, the ease in handling genetically and fermentation industrially, simple up-scaling, and simple down-stream processing, which have made them a champion as the hosts for biotechnology, recent challenges include creation of a “minimum genome factory”, in which as many genes as possible that are unnecessary for biosynthesis of a certain compound in a comfortable fermentor are deleted from the bacterial genomes. This is a national project in Japan and target microorganisms are *B. subtilis*, *E. coli*, *C. glutamicum* and yeasts including *S. cerevisiae* and *Schizosaccharomyces pombe*. Such

minimum genome factories may be very useful for production of some compound classes with few contaminants, although certain compounds are not efficiently transported across the membrane or exert toxicity on the hosts. Furthermore, it is useful to use eukaryotic microorganisms, such as yeasts and fungi, to express a step containing a membrane-bound and specific electron-transfer system-requiring enzyme, as we showed for IFS in production of genistein. Although transgenic approaches in plants, as well as in microorganisms, undoubtedly contribute to agricultural productivity, food quality, crop improvement, and human health, commercial production of transgenic plants or the compounds derived from "genetically modified organisms (GMO)" depends completely on public acceptance.

Flavonoid and stilbenoid biosynthetic pathways as secondary metabolism in plants have presumably evolved over billions of years, as have evolved antibiotic biosynthetic pathways in actinobacteria. It takes billions of years for plants and actinobacteria to reconstitute a pathway for a certain compound. It takes only a week or 10 days for us to assemble pathways for production of flavonoids, stilbenoids and curcuminoids in *E. coli* and generate derivatives or analogs of these complex plant-specific polyketides by adding decorating enzymes from various organisms, so that they can be used clinically. I dare to say that the *E. coli* cells have been endowed with the ability of secondary metabolism (Fig. 10). This concept and approach can be applied to any other compounds as a target molecule and any microorganisms as a host. However, as long as we mimic some biosynthetic pathways that Mother Nature has assembled genes for constructing them for billions of years, this approach probably will not work for generating wholly novel compounds. I agree with H. G. Floss [77] in that combinatorial biosynthesis/metabolic engineering should be viewed as a complement to, not a substitute for, the screening for natural product libraries and extracts.

Acknowledgments This work was supported by a research grant from the Noda Institute for Scientific Research, a grant from the New Energy and Industrial Technology Development Organization of Japan, and a Grant-in-Aid for Scientific Research on Priority Area "Applied Genomics" from Monkasho.

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