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Biosynthesis of Natural Flavanones in *Saccharomyces cerevisiae*

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A four-step flavanone biosynthetic pathway was constructed and introduced into *Saccharomyces cerevisiae*. The recombinant yeast strain was fed with phenylpropanoid acids and produced the flavanones naringenin and pinocembrin 62 and 22 times more efficiently compared to previously reported recombinant prokaryotic strains. Microbial biosynthesis of the flavanone eriodictyol was also achieved.

Flavonoids are a diverse family of plant polyphenols that demonstrate great potential in the treatment of various human pathological conditions (1, 10). The health-promoting effects of flavonoids have stimulated significant research toward the elucidation of their biosynthetic networks, as well as the development of production platforms using well-characterized hosts (8, 19). Within this context, Hwang et al. recently described recombinant *Escherichia coli* strains that lead to the synthesis of flavanones, the common precursors of the vast majority of flavonoids (7).

In plants, flavanone biosynthesis begins with the hydroxylation of cinnamic acid to *p*-coumaric acid by a membrane-bound P450 monooxygenase, cinnamate 4-hydroxylase (C4H) (Fig. 1). *p*-Coumaric acid is then activated to *p*-coumaroyl-coenzyme A (CoA) by 4-coumaroyl:CoA ligase. In the next step, three molecules of malonyl-CoA are sequentially added to one molecule of *p*-coumaroyl-CoA, yielding tetrahydroxychalcone, by the action of a polyketide synthase, chalcone synthase. Finally, chalcone isomerase converts the C_{15} compound tetrahydroxychalcone into (2*S*)-flavanones (Fig. 1) (5).

We present here the construction of a gene cluster that contains four plant-derived genes of the early flavonoid biosynthetic pathway that allows the conversion of phenylpropanoid acids into flavanones in *Saccharomyces cerevisiae*. This resulted in a substantial increase in the amount of flavanones produced compared to recombinant *E. coli* production and opens the possibility of producing several other flavonoid molecules whose biosynthesis requires the action of plant P450 monooxygenases.

Cloning of flavanone pathway in yeast. Vector YEplac181 was utilized for cloning the flavanone biosynthetic gene cluster in *S. cerevisiae*. This cluster included four structural genes of heterologous plant origins: *C4H* cDNA from *Arabidopsis thaliana* (2), isolated from expressed sequence tag clone RAFL06-11-J16 (RIKEN BioResource Center) (14, 15); *Pc4cL-2* cDNA from *Petroselinum crispum* (GenBank accession number AF233638) isolated from parsley young leaves by reverse transcription-PCR (RT-PCR) (9); *CHI-A* and *chs* cDNAs from *Petunia* × *hybrida* (GenBank accession numbers X14589 and

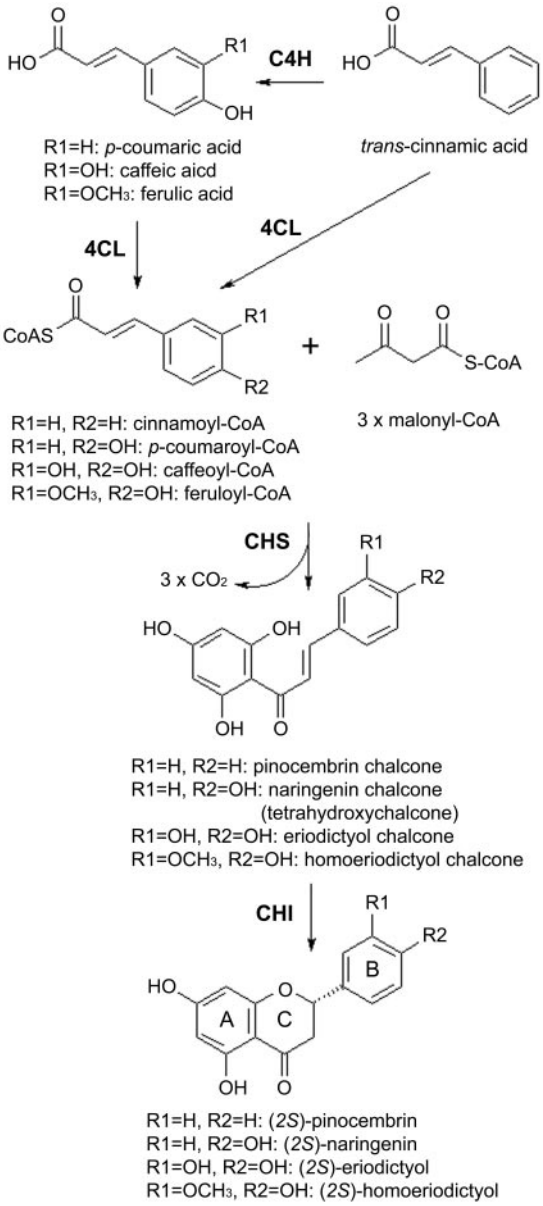

FIG. 1. Flavanone biosynthesis in plants. Abbreviations: C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase.

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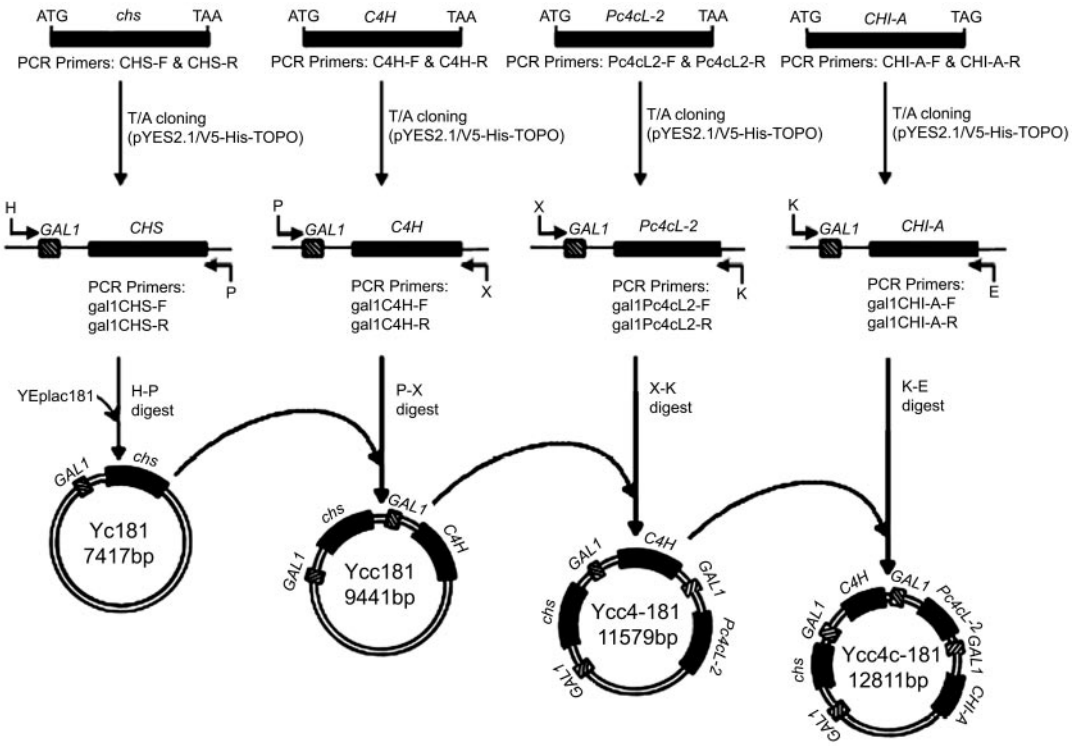

FIG. 2. Schematic representation of the cloning strategy used for assembling plasmid Ycc4c181, carrying the four flavanone biosynthetic genes. Abbreviations for restriction enzymes: H, HindIII; P, PstI; X, XbaI; K, KpnI; E, EcoRI. The PCR and RT-PCR primer sequences used are presented in Table 1.

X13225, respectively) isolated from petunia corolla also by RT-PCR (16, 17). We chose parsley 4-coumaroyl:CoA ligase-2 because this enzyme has been documented to accept caffeic acid, ferulic acid, and cinnamic acid with 28%, 66%, and 21% efficiency, respectively, in addition to its natural substrate, *p*-coumaric acid (9). We chose *CHI-A* because its previous cloning and expression in tomato plants led to a significant increase in the accumulation of flavanones (11).

Cloning of the four-gene cluster in yeast was accomplished by following a cloning strategy that has previously been utilized successfully for *E. coli* (19). In the first step, each gene is cloned separately under the control of a species-specific promoter (in the present study, the strong *S. cerevisiae GAL1* promoter). In the second step, each gene is amplified together with the promoter and is cloned using restriction digestions into the plasmid of choice (in the present study, *S. cerevisiae* plasmid YEplac181) (Fig. 2 and Table 1).

The successful application of this strategy in *E. coli* cannot be considered a precedent for success in yeast. This is because *S. cerevisiae* tends to be more recombination prone, and as a

TABLE 1. Primers used in this study and referred to in Fig. 1

Primer	Sequence (5′–3′)[a]
C4H-F	**ATG**GACCTCCTCTTGCTGGAGAAGT
C4H-R	*TTA*ACAGTTCCTTGGTTTC**TA**ACGATTATGGAGTGG
Pc4cL2-F	**ATG**GGAGACTGTGTAGCACCCAAAG
Pc4cL2-R	*TTA*TTTGGGAAGATCACCGGATGC
CHS-F	**ATG**GTGACAGTCGAGGAGTATCGTA
CHS-R	*TTA*AGTAGCAACACTGTGGAGGACA
CHI-A-F	**ATG**TCTCCTCCAGTGTCCGTTACTA
CHI-A-R	*CTA*GACTCCAATCACTGGAATAGTAGATTTCTCGG
gal1C4H-F	GGGGCTGCAGACGGATTAGAAGCCGCCGAG
gal1C4H-R	CCCCTCTAGA*TTA*ACAGTTCCTTGGTTTCATAACGATTATGGAGTGG
gal1Pc4cL2-F	GGGGTCTAGAACGGATTAGAAGCCGCCGAG
gal1Pc4cL2-R	CCCCGGTACC*TTA*TTTGGGAAGATCACCGGATGC
gal1CHS-F	GGGGAAGCTTACGGATTAGAAGCCGCCGAG
gal1CHS-R	CCCCCTGCAG*TTA*AGTAGCAACACTGTGGAGGACA
gal1CHI-A-F	GGGGGGTACCACGGATTAGAAGCCGCCGAG
gal1CHI-A-R	CCCCGAATTC*CTA*GACTCCAATCACTGGAATAGTAGATTTCTCGG

[a] Underlining indicates restriction enzyme cleavage sites, corresponding to the primer description. Boldface indicates a start codon, and italics indicate a stop codon.

TABLE 2. Retention times of standard (authentic) compounds used in the present study

Compound	Retention time (min)
Pinocembrin	16.3
Naringenin	12.8
Eriodictyol	11.1
Homoeriodictyol	12.9

result, tandemly repeated copies of DNA (such as the 451-bp *GAL1* promoter) have a higher probability of being looped out by excisional recombination (12). Therefore, the stability of the insertions must be carefully determined (3, 18). For that purpose, we grew the recombinant yeast strain for a maximum of 65 h at 30°C in leucine minimal selection medium (SC-Leu minimal medium) (4). Culture samples were taken every 24 h, and after plasmid isolation, the presence of each of the four genes together with the *GAL1* promoter was tested by restriction digestions and PCRs. No recombination events that would lead to the loss of a gene(s) or promoters were observed during the 65-h time frame.

Exploring flavanone biosynthesis in *S. cerevisiae*. We tested the ability of the recombinant yeast strain to synthesize flavanone compounds by feeding it with phenylpropanoid acids, such as cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid. In order to reduce cell growth inhibition, phenylpropanoid precursors were added every 13 h to the cultures in five equal doses, reaching a final concentration of 1 mM. In all cases, cultures were terminated after 65 h of incubation and flavonoid substances were extracted from the culture broth with an equal volume of ethyl acetate. They were further analyzed by reverse-phase high-performance liquid chromatography (HPLC) using an acetonitrile-water gradient, at a flow rate of 1.0 ml/min. The HPLC conditions were as follows: 10 to 40% for 10 min, 40 to 60% for 5 min, and 60 to 10% for 2 min. The retention times under these HPLC conditions for the standard authentic samples are presented in Table 2.

When cinnamic acid was used as a precursor metabolite and galactose as the sole carbon source and inducer, a large amount (16.3 mg/liter) of the corresponding unhydroxylated flavanone pinocembrin accumulated in the medium. This is a 22-fold increase compared to the amount of pinocembrin produced by the most efficient *E. coli* recombinant strain (7). However, only a relatively low concentration of naringenin (0.2 mg/liter) was detected, demonstrating that although C4H was functionally expressed in yeast, it is still a rate-limiting step enzyme in the four-enzyme hybrid pathway. It is possible that increasing the activity of CPR1, the yeast P450 reductase that is required for P450 monooxygenase function through episomal overexpression, could lead to increased C4H activity (6). When *p*-coumaric acid was used as a precursor, a large amount of naringenin (28.3 mg/liter) accumulated in the culture, which is 62 times higher compared to the amount of naringenin produced by the most efficient recombinant *E. coli* strain. Similarly, when caffeic acid was used as a precursor, natural (2*S*)-eriodictyol was produced in significant amounts (6.5 mg/liter). This is the first time eriodictyol biosynthesis has been achieved through microbial fermentation. Finally, ferulic acid, which carries a methoxy group on the aromatic ring, failed to be metabolized by the recombinant yeast strain. This result is in agreement with recent data obtained by Schroeder et al. demonstrating that *O*-methylations on the B ring of flavonoid substrates result in complete loss of enzymatic activity. It is therefore possible that B-ring methylations occur later in the complex flavonoid pathway to the end products (i.e., after flavanones have been synthesized) (13). The biotransformation results are summarized in Table 3.

TABLE 3. Production of natural flavanones by recombinant yeast *S. cerevisiae* INVSCI harboring plasmid Ycc4c181[a]

Acid converted	Production (mg/liter) of:			
	Pinocembrin	Naringenin	Eriodictyol	Homoeriodictyol
Cinnamic acid	16.3	0.2	NA[b]	NA
p-Coumaric acid	NA	28.3	NA	NA
Caffeic acid	NA	NA	6.5	NA
Ferulic acid	NA	NA	NA	0

[a] Cultures were grown in SC-Leu minimal medium with galactose as a carbon source and inducer.
[b] NA, not applicable.

In conclusion, we describe the biosynthesis of milligram quantities of flavanone substances from an *S. cerevisiae* recombinant strain that carries a plant-derived gene cluster. Since many flavonoid substances are formed through the action of P450 monooxygenases that cannot be readily expressed in *E. coli*, our success in producing a variety of flavanone skeletons from recombinant yeast will allow us to proceed in the future with the biosynthesis of several other high-value flavonoid molecules, such as genistein and quercetin.

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