Metabolic Engineering of the Phenylpropanoid Pathway in *Saccharomyces cerevisiae*

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Flavonoids are valuable natural products derived from the phenylpropanoid pathway. The objective of this study was to create a host for the biosynthesis of naringenin, the central precursor of many flavonoids. This was accomplished by introducing the phenylpropanoid pathway with the genes for phenylalanine ammonia lyase (PAL) from *Rhodosporidium toruloides*, 4-coumarate:coenzyme A (CoA) ligase (4CL) from *Arabidopsis thaliana*, and chalcone synthase (CHS) from *Hypericum androsaemum* into two *Saccharomyces cerevisiae* strains, namely, AH22 and a *pad1* knockout mutant. Each gene was cloned and inserted into an expression vector under the control of a separate individual *GAL10* promoter. Besides its PAL activity, the recombinant PAL enzyme showed tyrosine ammonia lyase activity, which enabled the biosynthesis of naringenin without introducing cinnamate 4-hydroxylase (C4H). 4CL catalyzed the conversion of both *trans*-cinnamic acid and *p*-coumaric acid to their corresponding CoA products, which were further converted to pinocembrin chalcone and naringenin chalcone by CHS. These chalcones were cyclized to pinocembrin and naringenin. The yeast AH22 strain coexpressing PAL, 4CL, and CHS produced approximately 7 mg liter⁻¹ of naringenin and 0.8 mg liter⁻¹ of pinocembrin. Several by-products, such as 2',4',6'-trihydroxydihydrochalcone and phloretin, were also identified. Precursor feeding studies indicated that metabolic flux to the engineered flavonoid pathway was limited by the flux to the precursor L-tyrosine.

Flavonoids are a class of widely distributed water-soluble plant pigments derived from the phenylpropanoid pathway, with more than 6,000 identified so far (13). In addition to their in planta function of protecting plants from UV irradiation and attacks by fungi and animals (13), they have also been shown to possess anti-inflammatory, antiallergenic, and antioxidant activities in humans (3, 6, 13, 24, 26). Many flavonoids are reported to possess activities against certain cancer types, such as skin cancer, breast cancer, and colon cancer (4, 8, 11, 22, 29). Studying the effects of specific flavonoids requires their purification from plant tissue, which is often difficult because of the low concentrations of certain flavonoids and numerous similar natural products. The productivity of flavonoids is also limited by the low growth rates of plants. The chemical synthesis of flavonoids can be achieved from simple starting materials. However, extreme reaction conditions and toxic chemicals are required (12). Therefore, the transfer of plant metabolic pathways into heterologous hosts such as bacteria or *Saccharomyces cerevisiae* is an attractive alternative source of flavonoids.

Several groups have recently begun to reconstitute the early steps of the phenylpropanoid pathway in microbes such as *Escherichia coli* (17, 37) and *Saccharomyces cerevisiae* (30). In plants, the biosynthesis of naringenin, the central precursor of most flavonoids, involves the following five enzymes in the phenylpropanoid pathway: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:coenzyme A (CoA) ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI) (Fig. 1). PALs from some plants, for

example, *Zea mays* L., also have tyrosine ammonia lyase (TAL) activity, converting tyrosine to *p*-coumaric acid (Fig. 1), the substrate of 4CL (31). Since tyrosine already has a 4-hydroxyl group, this pathway bypasses C4H. In addition, naringenin chalcone cyclizes in acidic solution without requiring the enzyme CHI (14, 27). The production of flavanones in genetically engineered *E. coli* was first reported by Hwang et al. (17). In their study, an artificial gene cluster containing PAL, 4CL, and CHS was constructed, and *E. coli* cells expressing these three enzymes produced two flavanones, pinocembrin and naringenin. Recently, Watts and coworkers cloned a bacterial TAL gene which was coexpressed with 4CL and CHS in *E. coli*. Their study showed that naringenin production could reach levels as high as 20.8 mg liter⁻¹ (37). Ro and Douglas connected the first two enzymes in *S. cerevisiae* by coexpressing *Populus* PAL, C4H, and cytochrome P450 reductase. They evaluated the carbon flux through the multienzyme system from phenylalanine to *p*-coumaric acid in yeast (30).

In this study, we describe the production of naringenin and pinocembrin by construction of the phenylpropanoid pathway in the yeast *S. cerevisiae*. We chose *S. cerevisiae* as the host because it has some advantages over *E. coli* for expressing certain eukaryotic heterologous proteins. We hypothesized that plant enzymes would be better expressed in a eukaryotic host since yeast is capable of performing posttranslational modifications of the eukaryotic proteins. In addition, yeast has similar intracellular compartments to those of plant cells. Furthermore, several cytochrome P450 (CYP) enzymes are involved in flavonoid biosynthesis, and yeast has been shown by several groups to be an excellent host for in vivo CYP activity (16, 19, 28, 33, 36). One reason for this is the presence of an

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FIG. 1. Proposed reactions catalyzed by *S. cerevisiae* AH22 coexpressing PAL, 4CL, and CHS. PAL (TAL), phenylalanine (tyrosine) ammonia lyase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; Pad1p, phenylacrylic acid decarboxylase. Overexpressed proteins are shown in bold. The biosynthesis of naringenin in plants is shown in the box.

endoplasmic reticulum, which is where CYP and CYP reductase are targeted in plants.

We inserted *PAL* from the red yeast *Rhodospiridium toruloides*, *4CL* from the plant *Arabidopsis thaliana*, and *CHS* from the plant *Hypericum androsaemum* into a yeast expression vector. Each gene was under the control of its own galactose-inducible promoter. Yeast harboring this vector produced naringenin and pinocembrin through the phenylpropanoid pathway, as well as four by-products, two of which were identified as phloretin and 2',4',6'-trihydroxydihydrochalcone through a sequential side reaction (Fig. 1). To our knowledge, this is the first study with a successful synthesis of flavonoids in a heterologous eukaryotic system.

MATERIALS AND METHODS

Chemicals. Naringenin, pinocembrin, and phloretin were purchased from Sigma-Aldrich (St. Louis, Mo.). 2',4',6'-Trihydroxydihydrochalcone was purchased from Apin Chemicals (Abingdon, Oxon, United Kingdom). All other chemicals were obtained at the highest available purity and were used without further purification.

Microbial strains and plasmids. *E. coli* DH5 α -T1 and OmniMAX-T1 were purchased from Invitrogen (Carlsbad, Calif.) and used for bacterial transformation. *E. coli* strains were cultured at 37°C in Luria-Bertani medium containing 1% tryptone, 0.5% yeast extract, and 1% NaCl. Ampicillin (100 mg liter⁻¹) was added to the Luria-Bertani medium for culturing *E. coli* that harbored the cloning or expression vector.

The plasmids pCR2.1-TOPO and pYES2.1/V5-His-TOPO (Invitrogen, Carlsbad, Calif.) were used to clone *PAL*, *CHS*, and *4CL*. The plasmid pKS2 μ Hyg was a gift from N. Ho (LORRE, Purdue University) and was used as the expression vector for *PAL*, *4CL*, and *CHS*.

S. cerevisiae AH22 (*MATa leu2-3 leu2-112 his4-519 can1*) (ATCC 38626) and

a *pad1* knockout (*MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 Δ PAD1*) (ATCC 4005833) (38) were used as hosts for enzyme expression.

Enzymes and structural genes. The Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, Ind.) and *Taq* DNA polymerase (Brinkmann, Westbury, N.Y.) were used for PCR. T4 DNA ligase and the restriction enzyme KpnI were purchased from New England Biolabs. All other restriction enzymes were purchased from Invitrogen (Carlsbad, Calif.). The *PAL* cDNA (GenBank accession no. AX366866) from *R. toruloides* was provided by BioMarin Pharmaceutical Inc. (Novato, Calif.). *4CL* (GenBank accession no. U18675) from *A. thaliana* was a gift from C. Chapple (Biochemistry, Purdue University). *CHS* (GenBank accession no. AF315345) from *H. androsaemum* was generously provided by L. Beerhues (Institute of Pharmaceutical Biology, Germany).

Construction of plasmids. For the construction of pKS2 μ Hyg-PAL, the promoter DNA of *GAL10* (GenBank accession no. K02115), the terminator gene *XKS1* (GenBank accession no. X61377) (both provided by N. Ho [LORRE, Purdue University]), and the *PAL* gene were amplified by PCR using the Expand High Fidelity PCR system with the primer pairs 5-GAL10-ApaI/3-GAL10-PALF, 5-PAL-GAL10F/3-PAL-XKF, and 5-XK-PALF/3-XK-ApaI (Table 1). Each PCR product was purified with a QIAEX II gel extraction kit (QIAGEN, Valencia, Calif.). Next, the termination sequence *XKS1* and the *PAL* gene were connected by overlap extension PCR as described elsewhere (15), using the primer pair 5-PAL-GAL10F/3-XK-ApaI; the PCR product PAL-XK and the promoter *GAL10* were then connected by overlap extension PCR using the primer pair 5-GAL10-ApaI/3-XK-ApaI and *Taq* DNA polymerase. The resulting PCR product, GAL10-PAL-XK, was cloned into the plasmid pCR2.1-TOPO by using the TOPO cloning technology (Invitrogen manual, Invitrogen, Carlsbad, Calif.). Finally, the fragment GAL10-PAL-XK was cloned into the shuttle vector pKS2 μ Hyg at the ApaI site, resulting in pKS2 μ Hyg-PAL.

Using the same procedure as that described for the construction of pKS2 μ Hyg-PAL and choosing the appropriate pairs of primers (Table 1), we constructed the PCR product GAL10-4CL-XK. This PCR product was cloned into the plasmid pYES2.1/V5-His-TOPO by using the TOPO cloning technology. The plasmid pKS2 μ Hyg-4CL was constructed by inserting GAL10-4CL-XK into the vector pKS2 μ Hyg at the BamHI site.

TABLE 1. Primers used for construction of *PAL*, *4CL*, and *CHS* expression vectors

Primer	Sequence (5' to 3') ^a	Restriction site
<i>PAL</i> primers		
5-GAL10-ApaI	TTTCTCCTTGAGGGCCAGTATAGAGG	ApaI
3-GAL10-PALF	GCGAGATCGAGTCGAGCGAGGGTGCCATTATATTGAATTTTCAAAAATTCTTAC	
5-PAL-GAL10F	AAGAATTTTGGAAAATTCAATATAAAATGGCACCCTCGCTCGACTCGATCTCGC	
3-PAL-XKF	TGAGAGTCTTTCCAGTTCGTCATCTAAGCGAGCATCTTGAGGAGGACG	
5-XK-PALF	CCTCCTCAAGATGCTCGCTTAGATGACGAAGTGGAAAGACTCTCATC	
3-XK-ApaI	GATGATTAAACAGGGCCCTAGCTCTTTCAATGC	ApaI
<i>4CL</i> primers		
5-GAL10-BamHI	TTTCTCCTTGAGGATCCAGTATAGAGG	BamHI
3-GAL10-4CLF	GAGAAACTGCTTGTTCTTGTTGGCGCCATTATATTGAATTTTCAAAAATTCTTAC	
5-4CL-GAL10F	AAGAATTTTGGAAAATTCAATATAAAATGGCGCCACAAGAACAAGCAGTTTCTCAGG	
3-4CL-XKF	TGAGAGTCTTTCCAGTTCGTCATTCACAATCCATTTGCTAGTTTTGCC	
5-XK-4CLF	GAGGGCAAAACTAGCAAATGGATTGTGAATGACGAAGTGGAAAGACTCTCATC	
3-XK-BamHI	GATGATTAAACAGGATCCCTAGCTCTTTCAATGC	BamHI
<i>CHS</i> primers		
5-GAL10-KpnI	TTTCTCCTTGAGGTACCAGTATAGAGG	KpnI
3-GAL10-CHSF	TCCTGACTTCTTCCACGGTCACCAATTATATTGAATTTTCAAAAATTCTTAC	
5-CHS-GAL10F	AAGAATTTTGGAAAATTCAATATAAAATGGTGACCGTGGAAAGTCAAGGAGGCGC	
3-CHS-XKF	TGAGAGTCTTTCCAGTTCGTCATCTAGTTAATGGCGACACTGTGAAGG	
5-XK-CHSF	TGTCCTTCACAGTGTGCGCCATTAAGTAGATGACGAAGTGGAAAGACTCTCATC	
3-XK-KpnI	GATGATTAAACAGGTACCTAGCTCTTTCAATGC	KpnI

^a Underlining indicates a restriction site. Bold italics indicate a start or stop codon.

The plasmid pKS2 μ Hyg-PAL was digested with ApaI, and then the fragment GAL10-PAL-XK was inserted into the plasmid pKS2 μ Hyg-4CL, which had been predigested with ApaI, resulting in the plasmid pKS2 μ Hyg-PAL-4CL.

GAL10-CHS-XK was cloned into the plasmid pCR2.1-TOPO as described for the construction of pKS2 μ Hyg-PAL by choosing appropriate primers (Table 1). GAL10-CHS-XK was excised from the plasmid pCR2.1-TOPO by the use of KpnI and then subcloned into pKS2 μ Hyg at the KpnI site.

GAL10-CHS-XK was excised from the plasmid pCR2.1-TOPO by the use of KpnI and then subcloned into pKS2 μ Hyg-PAL-4CL at the KpnI site. Thus, the three genes, *PAL*, *4CL*, and *CHS*, were cloned into the pKS2 μ Hyg vector together with their own *GAL10* promoters and terminator genes.

E. coli OmniMAX-T1 was transformed with the pKS2 μ Hyg-PAL-4CL-CHS plasmid. For the other plasmids, *E. coli* DH5 α -T1 was used as the host.

All expression vectors constructed for this study are listed in Table 2.

Yeast transformation. The transformation of *S. cerevisiae* AH22 or the *S. cerevisiae* *pad1* knockout strain was performed by the lithium acetate/SS carrier DNA/polyethylene glycol method (9). Yeast transformants were selected on YPD plates (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) containing 200 mg liter⁻¹ hygromycin (A.G. Scientific, San Diego, Calif.) and/or 200 mg liter⁻¹ Geneticin (Life Technologies, Inc., Rockville, Md.).

Yeast cultivation. Single colonies of the transformed yeast strain AH22 containing pKS2 μ Hyg-PAL, pKS2 μ Hyg-4CL, pKS2 μ Hyg-PAL-4CL, pKS2 μ Hyg-CHS, or pKS2 μ Hyg-PAL-4CL-CHS were inoculated into 10 ml of YPD medium containing 200 mg liter⁻¹ hygromycin at 30°C and shaken at 300 rpm for about 20 h. An appropriate volume of cells was added to 25 ml of fresh YPD medium with 200 mg liter⁻¹ hygromycin in a flask to reach an optical density at 600 nm of 0.05. After 19 h of growth, the cells were centrifuged at 1,000 $\times$ g for 5 min and the supernatant was discarded. The cells were resuspended in 25 ml of YPL medium (1% yeast extract, 2% peptone, and 2% galactose) containing 200 mg liter⁻¹ hygromycin. Induction was continued for up to 40 h. Cultivation of the

pad1 knockout yeast mutant containing the expression vectors was the same as that of AH22, except that an additional 200 mg liter⁻¹ of Geneticin was added to the YPD or YPL medium.

Crude protein extract preparation. After 6 h of induction, AH22 cells expressing 4CL were centrifuged at 3,000 $\times$ g for 5 min at 4°C. An appropriate volume of CellLytic-Y yeast cell lysis/extraction reagent (Sigma, St. Louis, Mo.) was added to the cell pellet, resulting in a mixture of 3 ml of CellLytic-Y reagent per gram of yeast cell pellet. The mixture was gently shaken for 20 min at 4°C. Afterwards, the lysed cells were centrifuged for 10 min at 12,000 $\times$ g, and the supernatant containing the crude protein extract was stored at -80°C or put on ice for immediate use.

HPLC method. Cinnamic acid and *p*-coumaric acid were analyzed by high-performance liquid chromatography (HPLC) on an SB-C₁₈ column (4.6 mm $\times$ 75 mm; Agilent) by the following method. Solvent A was 1.5% (vol/vol) acetic acid in water; solvent B was acetonitrile. The flow rate was 0.9 ml/min and the column was kept at 30°C. After 10 μ l of sample was injected, solvent B was kept at 5% for 4 min, then increased to 45% over 8.5 min, and finally held for 1 min. The solvent was returned to 5% solvent B over 2.5 min. Cinnamic acid and *p*-coumaric acid were quantified by the peak areas at 278 and 314 nm, respectively.

Naringenin, pinocembrin, phloretin, and 2',4',6'-trihydroxydihydrochalcone were analyzed by HPLC on the same C₁₈ column by use of a modified solvent gradient. Solvent B was kept at 5% for 4 min, then increased to 50% over 46 min, and finally held for 5 min before it was returned to 5% over 5 min. The injection volume was 20 μ l. The UV absorbance at 290 nm was recorded. All other conditions were the same as those described above.

Enzyme assays. The production of *trans*-cinnamic acid and *p*-coumaric acid by *S. cerevisiae* AH22 expressing PAL in YPL medium was also monitored by HPLC during the induction period. Samples were removed and centrifuged at 18,000 $\times$ g for 2 min. Each supernatant was mixed with an equal volume of methanol before HPLC injection.

In vitro 4CL activity was measured by incubating the crude protein extracted from AH22 expressing 4CL or from AH22 coexpressing PAL and 4CL with *p*-coumaric acid and CoA as substrates. A UV-visible light spectrophotometer was used to monitor the increase in *p*-coumaroyl-CoA at 333 nm (20, 39). The extinction coefficient for *p*-coumaroyl-CoA is 21 cm mM⁻¹ (35).

Flavonoid extraction and assay. After 6 to 40 h of induction, cells were centrifuged and the supernatant of AH22 or the *pad1* knockout strain expressing PAL, 4CL, and CHS was removed and mixed with an equal volume of methanol. The samples were then analyzed by HPLC. To investigate the possible presence of naringenin chalcone in the supernatant, we lyophilized the supernatant (5 ml)

TABLE 2. Expression vectors constructed for this study

Plasmid	Structural gene
pKS2 μ Hyg-PAL	<i>PAL</i> (<i>TAL</i>)
pKS2 μ Hyg-4CL	<i>4CL</i>
pKS2 μ Hyg-PAL-4CL	<i>PAL</i> (<i>TAL</i>), <i>4CL</i>
pKS2 μ Hyg-CHS	<i>CHS</i>
pKS2 μ Hyg-PAL-4CL-CHS	<i>PAL</i> (<i>TAL</i>), <i>4CL</i> , <i>CHS</i>

FIG. 2. Production of cinnamic acid (Δ) and *p*-coumaric acid ($\blacksquare$) by *S. cerevisiae* AH22-PAL during induction in YPL medium. The data are means $\pm$ standard deviations ($n = 3$).

after 40 h of induction. The dried powder was mixed with 2 ml of 62.5% methanol and 0.5 ml of 6 M HCl and then incubated at 90°C for 60 min (14, 27). Extracts were mixed with 2.5 ml of methanol and sonicated for 5 min. To investigate the intracellular phenylpropanoid accumulation after 40 h of induction, we collected yeast cells from 5 ml of culture by centrifugation at $3,000 \times g$ for 5 min. After the cells were lyophilized, we used two methods to extract naringenin. For methanol-HCl extraction, the procedure was similar to freeze-dried supernatant extraction. For direct methanol extraction, we added 70% methanol to the lyophilized cells and sonicated them at room temperature for 30 min (27). The extracts obtained from both methods were centrifuged at $18,000 \times g$ for 5 min before HPLC injection.

LC/MS and MS/MS. All electrospray ionization analyses were carried out on a FinniganMAT LCQ (ThermoFinnigan Corp., San Jose, Calif.) mass spectrometer system. For liquid chromatography-mass spectrometry (LC/MS), the same conditions as those described for the HPLC method were used for separation of the compounds. Both the positive and negative ion modes were applied, with the mass spectrometer scan ranging from 50 to 600 m/z . Tandem MS (MS/MS) results were obtained by selecting the ion of interest. The precursor ion was then subjected to collision-induced dissociation, resulting in the formation of product ions. The collision energy was set to 40% of the maximum available from the 5-V tickle voltage, with a 2-mass-unit isolation window.

RESULTS

To ensure the activity of each of the enzymatic steps, we constructed yeast strains expressing each of the genes, i.e., *PAL*, *4CL*, and *CHS*. The first two steps were investigated in strain AH22 expressing *PAL* and *4CL*. The final strain contained all three genes, for *PAL*, *4CL*, and *CHS*, in the same expression vector.

PAL (TAL) activity. *PAL* from *R. toruloides* catalyzes the conversion of both L-phenylalanine ($K_m = 0.29$ mM) and L-tyrosine ($K_m = 0.18$ mM) to their corresponding products (1). *PAL* was overexpressed in *S. cerevisiae* AH22 harboring pKS2 μ Hyg-PAL and tested for catalytic activity with L-phenylalanine and L-tyrosine as substrates. The production of *trans*-cinnamic acid and *p*-coumaric acid was analyzed by measuring the concentrations of these compounds in the complex medium YPL during the induction period (Fig. 2). As expected, both *PAL* and *TAL* activities were observed. The concentration of *trans*-cinnamic acid increased with the induction time until 6 h and then decreased. In contrast, the production of *p*-coumaric acid increased with the induction time from 0 to 10 h, maintained a level of about 25 to 30 $\mu\text{mol g cell}^{-1}$ until 26 h after induction, and then decreased. We observed that upon feeding of *trans*-cinnamic acid (100 μM) directly to AH22, it completely disappeared in 4 h, and the *p*-coumaric acid concentration (100 μM) decreased 40% in 12 h in AH22 yeast in YPD medium.

FIG. 3. In vitro 4CL activity assay for *S. cerevisiae* AH22 expressing 4CL ($\blacksquare$) and *S. cerevisiae* AH22 coexpressing PAL and 4CL (Δ). The control strain was *S. cerevisiae* AH22 ($\blacklozenge$).

4CL activity. To investigate the catalytic properties of 4-coumarate:CoA ligase, we extracted a crude protein mixture from AH22 yeast cells expressing 4CL or coexpressing PAL and 4CL after 6 h of induction. The in vitro 4CL activity was assayed by following the production of 4-coumaroyl-CoA, which absorbs at a wavelength of 333 nm. The results showed that 4-coumaroyl-CoA was produced in AH22 containing 4CL and in AH22 expressing both PAL and 4CL (Fig. 3), while no significant increase in the UV absorbance at 333 nm was detected with the control strain.

We also detected no *p*-coumaric acid accumulation in AH22 coexpressing PAL and 4CL in YPL medium during the induction period. To investigate the consumption of *p*-coumaric acid by AH22 expressing 4CL, we fed 100 μM *p*-coumaric acid to yeast after 4 h of induction. The concentration of *p*-coumaric acid was found to decrease dramatically with time in AH22 expressing 4CL or AH22 coexpressing PAL and 4CL, while the concentration was constant in the control strain (data not shown). Thus, both in vitro and in vivo analyses demonstrated that 4CL was actively expressed in *S. cerevisiae* AH22 harboring pKS2 μ Hyg-4CL or pKS2 μ Hyg-PAL-4CL.

Production of flavonoids in yeast strain AH22. Naringenin was produced in the yeast *S. cerevisiae* AH22 coexpressing PAL, 4CL, and CHS (Fig. 4A and 4B), while no naringenin was detected in the control AH22 strain, which was AH22 harboring pKS2 μ Hyg-CHS (Fig. 4C) or pKS2 μ Hyg-PAL-4CL (Fig. 4D). Pinocembrin, the product of cinnamoyl-CoA condensing with three molecules of malonyl-CoA, was detected in the cell extract after the cell pellet was lyophilized and extracted with methanol-HCl (Fig. 4B). This means that 4CL also catalyzed *trans*-cinnamic acid to cinnamoyl-CoA, which was further catalyzed to pinocembrin chalcone by CHS (Fig. 1). However, the amount of pinocembrin in the supernatant of the cell culture was too small to be detected.

Other than naringenin and pinocembrin, there were several other compounds produced by AH22 containing pKS2 μ Hyg-PAL-4CL-CHS (compare Fig. 4A and 4D). Two of them, 2',4',6'-trihydroxydihydrochalcone and phloretin (Fig. 4A and 4B), were identified by comparisons of their retention times during HPLC to those of authentic standards, and their molecular weights were confirmed by LC/MS (data not shown). The LC/MS results showed that the positive ions for the two unknown compounds were $[M + H]^+$ m/z 233 (U1) and m/z 217 (U2), respectively. This indicated that the molecular

FIG. 4. HPLC analysis of naringenin and pinocembrin synthesized by *S. cerevisiae* AH22 coexpressing PAL, 4CL, and CHS. (A) AH22 coexpressing PAL, 4CL, and CHS; (B) AH22 coexpressing PAL, 4CL, and CHS prepared by methanol-HCl cell extraction; (C) AH22 coexpressing CHS; (D) AH22 coexpressing PAL and 4CL. The samples for panels A, C, and D were supernatants of their corresponding cell cultures mixed with equal volumes of methanol. 1, naringenin; 2, pinocembrin; 3, phloretin; 4, 2',4',6'-trihydroxydihydrochalcone; U1, unknown compound 1; U2, unknown compound 2.

weights of these two compounds were 232 and 216, respectively. Further MS/MS results showed that U1 contained a phenol group and U2 contained a benzyl group. Hypothesized structures of U1 and U2 consistent with UV and MS analyses are shown in Fig. 1.

Interestingly, yeast expressing CHS alone also produced several compounds that were not found in AH22 or AH22 coexpressing PAL and 4CL. This demonstrated that CHS uses some intracellular metabolites in yeast as substrates. Currently, we have not identified these products.

The effect of induction time on the production of naringenin

FIG. 5. Production of naringenin by *S. cerevisiae* AH22 coexpressing PAL, 4CL, and CHS during induction. The data are means $\pm$ standard deviations ($n = 3$).

was also investigated. The concentration of naringenin increased with the induction time from 0 to 30 h and was maintained at about $5.8 \text{ mg liter}^{-1}$ until 40 h after induction (Fig. 5). Cell growth was also monitored during the induction period. Figure 5 shows that the cell mass stopped increasing after 30 h, suggesting that there was nutrient limitation thereafter. The average production level of naringenin in yeast reached $680 \pm 90 \mu\text{g g}^{-1}$ cell dry weight after 40 h of induction.

To investigate the possibility of naringenin chalcone in the supernatant, we examined conditions that would chemically convert chalcone to naringenin. We also injected the sample into the HPLC column at a neutral pH and did not detect naringenin chalcone, which indicated that the cyclization of naringenin chalcone preceded the acidic HPLC conditions. The average concentration of naringenin after acid treatment ($5.8 \pm 0.3 \text{ mg liter}^{-1}$) was similar to that without acid treatment ($5.4 \pm 0.4 \text{ mg liter}^{-1}$) (Table 3), which means that there was negligible naringenin chalcone in the supernatant. The intracellular concentration of naringenin was also analyzed. Two extraction methods were applied after the cells were lyophilized. The amount of naringenin extracted with methanol-HCl was about 25% larger than that extracted by methanol only (Table 3), which suggests that the cyclization reaction occurs inside yeast cells. The results also showed that the amount of intracellular naringenin was about 20% of the total naringenin (approximately 7 mg liter^{-1}) produced in the yeast culture after 40 h of induction (Table 3). The amount of pinocembrin produced by yeast was also quantified. Table 3 shows that the amount of pinocembrin extracted from yeast cells (0.8 to $\sim 0.9 \text{ mg liter}^{-1}$) was not affected by the extraction method. The concentrations of phloretin and 2',4',6'-trihydroxydihydrochalcone were approximately 9 mg liter^{-1} and 11 mg liter^{-1} , respectively (Table 3). Thus, the total amount of secreted products was about 17 mg liter^{-1} , with a maximum productivity of about $0.9 \text{ mg liter}^{-1} \text{ h}^{-1}$.

Precursor feeding study. To investigate whether flux to the aromatic amino acids or PAL activity was limiting, we compared *trans*-cinnamate and *p*-coumarate production in yeast overexpressing PAL in YPL medium versus that in YPL medium with the addition of 65 mg liter^{-1} of both L-tyrosine and L-phenylalanine. After 14 h of induction, the PAL overexpression strain produced 27% ($P < 0.01$) more *p*-coumarate in YPL medium with the addition of L-tyrosine and L-phenylala-

TABLE 3. Production of naringenin, pinocembrin, phloretin, and 2',4',6'-trihydroxydihydrochalcone by *S. cerevisiae* AH22 coexpressing PAL, 4CL, and CHS

Product	Amt in supernatant (mg liter ⁻¹)		Amt in cell extract (μg g ⁻¹ CDW)		Final total concentration (mg liter ⁻¹)
	MeOH ^a	MeOH/HCl ^b	MeOH ^c	MeOH/HCl ^d	
Naringenin	5.8 ± 0.3	5.4 ± 0.4	140 ± 10	180 ± 30 ^e	~7
Pinocembrin	ND	ND ^f	90 ± 10	110 ± 20	~0.8
Phloretin	7.1 ± 0.4	7.2 ± 0.3	200 ± 10	300 ± 40 ^g	~9
2',4',6'-Trihydroxydihydrochalcone	5.2 ± 0.3	4.4 ± 0.1	810 ± 60	970 ± 130	~11

^a The supernatant of the yeast culture was mixed with an equal volume of methanol before HPLC injection.

^b The supernatant of the yeast culture (5 ml) was freeze-dried and treated with a methanol-HCl solution.

^c The cell pellet (from 5 ml of yeast culture) was lyophilized and extracted with 70% methanol.

^d The cell pellet (from 5 ml of yeast culture) was lyophilized and extracted with a methanol-HCl solution.

^e $P < 0.1$.

^f ND, not detectable.

^g $P < 0.05$.

nine than in YPL medium. This result indicated that the L-tyrosine pool size is limiting for the production of *p*-coumarate in yeast overexpressing PAL alone. No statistically significant difference was observed for *trans*-cinnamate production, which was possibly due to competing reactions catalyzed by some endogenous yeast enzymes. We next examined whether the precursor limitations were similar for the entire engineered pathway. With yeast coexpressing PAL, 4CL, and CHS, we added 33 mg liter⁻¹ of *p*-coumarate or 65 mg liter⁻¹ of both L-tyrosine and L-phenylalanine to the YPL medium. Naringenin, phloretin, and U1 were increased 103% ($P < 0.05$), 52% ($P < 0.01$), and 61% ($P < 0.01$), respectively, in YPL medium containing 33 mg liter⁻¹ of *p*-coumarate, indicating that the expression levels of 4CL and CHS were sufficient to handle increased flux to *p*-coumarate. With the addition of L-tyrosine and L-phenylalanine, the HPLC results showed that phloretin and U1 were enhanced by 30% ($P < 0.01$) and 50% ($P < 0.05$), respectively, after 26 h, while no significant increase was observed for naringenin, U2, and 2',4',6'-trihydroxydihydrochalcone. These results indicated that the production of flavonoids in yeast overexpressing PAL, 4CL, and CHS was likely limited by L-tyrosine, not the enzyme expression levels.

Production of flavonoids in yeast *pad1* knockout strain. It has been reported that the endogenous yeast enzyme phenylacrylic acid decarboxylase (Pad1P, encoded by *PAD1*) is responsible for the decarboxylation of *trans*-cinnamic acid and *p*-coumaric acid (5, 10, 23) and perhaps the reduction of *trans*-cinnamic acid (23). We used the *pad1* knockout yeast as a host to produce flavonoids. We fed *trans*-cinnamic acid or *p*-coumaric acid exogenously to the *pad1* strain and observed no decrease in the concentration of either compound for 12 h in either YPD or YPL medium. For the *pad1* knockout strain overexpressing PAL alone, cinnamic acid accumulated and no degradation was detected (data not shown). However, it was surprising to see that in contrast to the case for AH22 overexpressing PAL, *trans*-cinnamic acid production was 10-fold higher than that of *p*-coumaric acid in the *pad1* knockout yeast overexpressing PAL (data not shown). We next investigated the suitability of the *pad1* host for expressing PAL, 4CL, and CHS. Unexpectedly, only phloretin, 2',4',6'-trihydroxydihydrochalcone, and U2 were detected after 36 h of induction (data not shown).

DISCUSSION

This study is the first report of the construction of the phenylpropanoid pathway for the synthesis of naringenin in yeast. Compared with the *E. coli* strain used in the study by Hwang et al. (17), our yeast strain, AH22 harboring pKS2μHyg-PAL-4CL-CHS, was able to produce >10-fold more naringenin. In addition, this yeast strain produced naringenin, pinocembrin, and four other products together in YPL medium. On the other hand, Hwang et al. controlled the entry point to the pathway by feeding the cells either tyrosine or phenylalanine. Therefore, compared to the case for the PAL-4CL-CHS pathway expressed in *E. coli*, our strain has significantly more flux into the phenylpropanoid pathway. In another study of flavonoid production in *E. coli*, a bacterial TAL enzyme which does not show PAL activity was coexpressed with 4CL and CHS, and high-level production of naringenin (20.8 mg liter⁻¹) was obtained in TB medium without feeding L-tyrosine to the cells (37). The production of phloretin was also observed in that study after feeding dihydro-*p*-coumaric acid to *E. coli* expressing 4CL and CHS (37). Our yeast strain produced a comparable concentration of secreted flavonoids (17 mg liter⁻¹) without considering the two unidentified products (U1 and U2) to that of the TAL-4CL-CHS proteins expressed in *E. coli*.

Naringenin and pinocembrin are derived from L-tyrosine and L-phenylalanine, respectively. PAL from *R. toruloides* catalyzes the deamination of both L-phenylalanine and L-tyrosine (1). Recombinant TAL expressed by *S. cerevisiae* AH22 harboring pKS2μHyg-PAL allows the phenylpropanoid pathway to bypass cinnamate, since L-tyrosine is converted directly from *p*-coumaric acid by deamination. Thus, in this study and others (17, 37), it was not required to express C4H to synthesize *p*-coumarate. Therefore, the flux to flavonoids is controlled by the pool sizes of L-phenylalanine and L-tyrosine and the enzyme selectivity of PAL (or TAL). Precursor feeding studies demonstrated that L-tyrosine limits the flux to flavonoid synthesis. Therefore, increasing the flux to L-tyrosine or using the bacterial TAL enzyme (37) would enhance the production of flavonoids.

Pad1P in *S. cerevisiae* was reported to decarboxylate *trans*-cinnamic acid and *p*-coumaric acid to their corresponding vinyl

derivatives (6, 10, 23). Styrene, the product of *trans*-cinnamic acid decarboxylation, was identified in *S. cerevisiae* overexpressing Pad1P (23). We also detected a small amount of styrene in an HPLC analysis of the AH22 strain that was fed cinnamic acid (data not shown). Larsson and coworkers also found that *trans*-cinnamic acid was converted to dihydrocinnamic acid (3-phenylpropanoic acid) in yeast overexpressing Pad1P but not in wild-type yeast (23). In contrast to this result, production of the dihydrochalcones in the *pad1* knockout yeast provided evidence that Pad1P is not the only enzyme involved in the reduction of *trans*-cinnamic acid and *p*-coumaric acid, if it is involved at all. The lower level of production of flavonoids derived from L-tyrosine by the *pad1* knockout strain expressing PAL-4CL-CHS was due to the low level of production of *p*-coumaric acid. However, the reason that the knockout of *PAD1* resulted in such a different PAL (TAL) behavior is unclear.

The production of phloretin and 2',4',6'-trihydroxydihydrochalcone suggests two possibilities. One possibility is that 4CL catalyzes the ligation of CoA with dihydrocinnamic acid and dihydro-*p*-coumaric acid to produce dihydrocinnamoyl-CoA and dihydro-*p*-coumaroyl-CoA, respectively. The other possibility is that 4CL ligates cinnamic and coumaric acids to their CoA esters, followed by reduction to their dihydro- derivatives by an uncharacterized yeast enzyme. CHS is a type III polyketide synthase that has broad substrate specificity (18, 32, 34). CHS from *H. androsaemum* condenses either *p*-coumaroyl-CoA or cinnamoyl-CoA with three molecules of malonyl-CoA to produce the corresponding chalcones (25). The identification of phloretin and 2',4',6'-trihydroxydihydrochalcone suggests that dihydrocinnamoyl-CoA and dihydro-*p*-coumaroyl-CoA are further catalyzed by CHS.

We hypothesize that the two unknown compounds (U1 and U2) with molecular weights of 232 and 216, respectively, are the products of dihydro-*p*-coumaroyl-CoA and dihydrocinnamoyl-CoA, respectively, condensing with two molecules of malonyl-CoA (Fig. 1). To confirm this, we performed MS/MS and found that each molecule produced fragments consistent with one phenol or benzyl group. 4-Hydroxy-6-(2-[4-hydroxyphenyl]ethyl)-pyran-2-one, a possible structure of U1, has not been reported before.

Several by-products, such as triketide lactone and tetra-ketide lactone, have been reported to be synthesized by CHS (2, 21). We speculated that these lactones may be present in yeast that expresses PAL, 4CL, and CHS. However, none of these potential products have been identified due to the lack of standards. CHS has also been reported to use various aliphatic CoA esters such as acetyl-CoA and butyryl-CoA as starter molecules (32, 34). Therefore, it was not surprising that multiple peaks were observed in the HPLC chromatograph for the yeast strain that expressed CHS alone (Fig. 4C). We are currently investigating the structures of these unidentified compounds.

The low 4CL activity with *trans*-cinnamic acid as a substrate may have contributed to the low-level production of pinocembrin. The 4CL enzyme used for this study was At4CL1 from *A. thaliana*, which catalyzes the conversion of *p*-coumaric acid, ferulic acid, and caffeic acid to their corresponding CoA products (7). Although 4CL also catalyzes *trans*-cinnamic acid to cinnamoyl-CoA, the K_m for *trans*-cinnamic acid was 6.3 mM,

significantly higher than that of *p*-coumaric acid (38 μ M). However, the V_{max} for *trans*-cinnamic acid was similar to that for *p*-coumaric acid (7). Another reason for the low 4CL activity was that *trans*-cinnamic acid was decarboxylated by Pad1P, which competed with 4CL.

Future work will focus on metabolic engineering strategies to increase the yield of naringenin and decrease the number of by-product flavonoids. Since *A. thaliana* C4H was previously successfully expressed in *S. cerevisiae* (28), the gene for C4H could be added to a recombinant yeast strain containing an integrated cytochrome P450 reductase, with the expression of PAL, 4CL, and CHS from our plasmid. We hypothesize that C4H would compete with Pad1P and/or the unknown endogenous yeast enzymes that degrade *trans*-cinnamic acid and redirect the carbon flux to *p*-coumaric acid to enhance the production of naringenin. The identification of the unknown enzyme(s) that is responsible for the reduction of *trans*-cinnamic acid or *p*-coumaric acid will enable us to further understand the engineered flavonoid biosynthesis pathway in yeast. These results will enable future efforts to produce several valuable flavonoids from naringenin.

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