Metabolic Engineering of the Phenylpropanoid Pathway in Saccharomyces cerevisiae

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Received 7 July 2004/Accepted 18 December 2004

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 Rhodospirillum toruloides, 4-coumarate:coenzyme A (CoA) ligase (4CL) from Arabidopsis thaliana, and chalcone synthase (CHS) from Hypericum androsaemum into two Saccharomyces cerevisiae strains, namely, AH122 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. These chalcones were cyclized to pinocembrin and naringenin. The yeast AH122 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 (14), 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 open reading frame which is transcribed from 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. These chalcones were cyclized to pinocembrin and naringenin. The yeast AH122 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.
endoplasmic reticulum, which is where CYP and CYP reductase are targeted in plants.

We inserted PAL from the red yeast Rhodosporidium toruloides, 4CL from the plant Arabidopsis thaliana, and CHS from the plant Hypericum androsaemenum into a yeast expression vector. Each gene was under the control of its own galactose-inducible promoter. Yeast harboring this vector produced naringenin in plants 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 DH5α-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 pKS2aHyg 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 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δpad1) (ATCC 4005833) (38) were used has hosts for enzyme expression.

Enzymes and structural genes. The Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, Ind.) and Taq DNA polymerase (Binkmann, 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. AX568666) 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. androsaemenum was generously provided by L. Beerhues (Institute of Pharmaceutical Biology, Germany).

Construction of plasmids. For the construction of pKS2aHyg-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-XK-ApaI and 5-GAL10-ApaI/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 GAL10 promoter were then connected by overlap extension PCR as described elsewhere (15), using the primer pair 5-GAL10-ApaI/3-XK-ApaI; 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; 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; using the TOPO cloning technology (Invitrogen manual, Invitrogen, Carlsbad, Calif.). Finally, the fragment GAL10-PAL-XK was cloned into the shuttle vector pKS2aHyg-Hyg at the ApaI site, resulting in pKS2aHyg-PAL.

Using the same procedure as that described for the construction of pKS2aHyg-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 (Invitrogen manual, Invitrogen, Carlsbad, Calif.). Finally, the fragment GAL10-4CL-XK was cloned into the shuttle vector pKS2aHyg-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 pKS2aHyg-PAL was transformed by electroporation into the vector pKS2aHyg at the BamHI site.
The plasmid pKS2aHyg-PAL was digested with ApaI, and then the fragment GAL10-PAL-XK was inserted into the plasmid pKS2aHyg-4CL, which had been predigested with ApaI, resulting in the plasmid pKS2aHyg-PAL-4CL. GAL10-CHS-XK was cloned into the plasmid pCR2.1-TOPO by the use of KpnI and then subcloned into pKS2aHyg at the KpnI site. Thus, the KpnI and then subcloned into pKS2aHyg-PAL-4CL at the KpnI site. Therefore, the plasmid pKS2aHyg-PAL-4CL was transformed with the pKS2aHyg-PAL-4CL-CHS, were cloned into the pCR2.1-TOPO by the use of KpnI and then subcloned into pKS2aHyg-PAL-4CL at the KpnI site. The three genes, PAL, 4CL, and CHS, were cloned into the pKS2aHyg vector together with their own GAL1 promoters and terminator genes.

### 4CL Primers

- **5'-GAL10-BamHI:** TTTTCTCCTGAGGATCCAGTATAGAGG
- **3'-GAL10-BamHI:** CCTTCTCCTGAGGATCCAGTATAGAGG
- **5'-PAL-BamHI:** GCCGAGCTCCAGTATAGAGG
- **3'-PAL-BamHI:** GGCGAGCTCCAGTATAGAGG
- **5'-KpnI-BamHI:** TTTTCTCCTGAGGATCCAGTATAGAGG
- **3'-KpnI-BamHI:** CCTTCTCCTGAGGATCCAGTATAGAGG

### C8H Primers

- **5'-GAL10-KpnI:** TTTTCTCCTGAGGATCCAGTATAGAGG
- **3'-GAL10-KpnI:** CCTTCTCCTGAGGATCCAGTATAGAGG
- **5'-PAL-KpnI:** GCCGAGCTCCAGTATAGAGG
- **3'-PAL-KpnI:** GGCGAGCTCCAGTATAGAGG
- **5'-KpnI:** TTTTCTCCTGAGGATCCAGTATAGAGG
- **3'-KpnI:** CCTTCTCCTGAGGATCCAGTATAGAGG

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Expression vectors constructed for this study</th>
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<tr>
<td>Plasmid</td>
<td>Structural gene</td>
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<tr>
<td>pKS2aHyg-PAL</td>
<td>PAL (TAL)</td>
</tr>
<tr>
<td>pKS2aHyg-4CL</td>
<td>4CL</td>
</tr>
<tr>
<td>pKS2aHyg-PAL-4CL</td>
<td>PAL (TAL), 4CL</td>
</tr>
<tr>
<td>pKS2aHyg-ChS</td>
<td>CHS</td>
</tr>
<tr>
<td>pKS2aHyg-PAL-4CL-ChS</td>
<td>PAL (TAL), 4CL, CHS</td>
</tr>
</tbody>
</table>

#### Table 1. Primers used for construction of PAL, 4CL, and CHS expression vectors

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5\’ to 3\’)</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-GAL10-Apal</td>
<td>TTTTCTCCTGAGG</td>
<td>ApaI</td>
</tr>
<tr>
<td>3'-GAL10-PALF</td>
<td>GCCGAGCTCCAGTATAGAGG</td>
<td>ApaI</td>
</tr>
<tr>
<td>5'-PAL-GAL10F</td>
<td>AAGAATTTGAATATTAATAAATGTTACAGTATAGAGG</td>
<td>ApaI</td>
</tr>
<tr>
<td>3'-PAL-XKF</td>
<td>TGAGAGCTCCAGTATAGAGG</td>
<td>ApaI</td>
</tr>
<tr>
<td>5'-XK-PALF</td>
<td>CCTTCTCCTGAGG</td>
<td>ApaI</td>
</tr>
<tr>
<td>3'-XK-Apal</td>
<td>GGAGGCAAAACTAGGAATATGTTAGT4ATGCG-AAACAGCTTCTCAGG</td>
<td>ApaI</td>
</tr>
</tbody>
</table>

A <sup>*</sup> Underlining indicates a restriction site. Bold italics indicate a start or stop codon.

### Enzyme Assays

- **In vitro 4CL activity:** Measured by incubating the crude protein extract from AH22 expressing 4CL or from AH22 coexpressing PAL and 4CL with p-coumaroyl-CoA at 333 nm (20, 39).
- **Restriction enzyme assay:** The production of p-coumaroyl-CoA was monitored 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 45% over 8.5 min, and finally held for 1 min. The solvent was returned to 5% solvent B over 2.5 min. Naringenin, pinocembrin, phloretin, and 2',4',6'-trihydroxydihydrochalcone were analyzed by HPLC on the same C18 column by use of a modified solvent gradient. The flow rate was 0.9 ml/min and the column was kept at 30°C. After 10 μl 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. 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 μl. The UV absorbance at 290 nm was recorded. Allo other conditions were the same as those described above.

### Flavonoid extraction and assay

- **Flavonoid extraction and assay:** After 6 to 40 h of induction, cells were centrifuged 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 quantified by high-performance liquid chromatography (HPLC) on an SB-C18 column (4.6 mm × 75 mm; Agilent) by the following method.**

### Conclusion

- **4CL activity:** Measured by incubating the crude protein extract from AH22 expressing 4CL or from AH22 coexpressing PAL and 4CL with p-coumaroyl-CoA at 333 nm (20, 39).

### References

- [Jiang ET AL. APPL. ENVIRON. MICROBIOL.](http://aem.asm.org)
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 × 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 × g for 5 min before HPLC injection.

LC/MS and MS/MS. All electrospray ionization analyses were carried out on a Finngan MAT LQTO (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 same 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_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 μmol/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.

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-coumaryl-CoA, which absorbs at a wavelength of 333 nm. The results showed that 4-coumaryl-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_Hyg-4CL or pKS2_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_Hyg-CHS (Fig. 4C) or pKS2_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_Hyg-PAL-4CL-CHS (compare Fig. 4A and D). Two of them, 2',4',6'-trihydroxydihydrochalcone and phloretin (Fig. 4A and B), 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...
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 was also investigated. The concentration of naringenin increased with the induction time from 0 to 30 h and was maintained at about 5.8 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 ± 90 \(\mu\)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 ± 0.3 mg liter\(^{-1}\)) was similar to that without acid treatment (5.4 ± 0.4 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 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 ~0.9 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 mg liter\(^{-1}\) h\(^{-1}\).

**Precursor feeding study.** To investigate whether flux to the aromatic amino acids orPAL activity was limiting, we compared \(\text{trans-}
\text{cinnamate}\) and \(\text{p-coumarate}\) production in yeast overexpressing PAL in YPL medium versus that in YPL medium with the addition of 65 mg liter\(^{-1}\) of both \(\text{l-tyrosine}\) and \(\text{l-phenylalanine}\). After 14 h of induction, the PAL overexpression strain produced 27% (\(P < 0.01\)) more \(\text{p-coumarate}\) in YPL medium with the addition of \(\text{l-tyrosine}\) and \(\text{l-phenylala-}
TABLE 3. Production of naringenin, pinocembrin, phloretin, and 2',4',6'-trihydroxydihydrochalcone by S. cerevisiae AH22 coexpressing PAL, 4CL, and CHS

<table>
<thead>
<tr>
<th>Product</th>
<th>Amt in supernatant (mg liter⁻¹)</th>
<th>Amt in cell extract (µg g⁻¹ CDW)</th>
<th>Final total concentration (mg liter⁻¹)</th>
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<tr>
<td></td>
<td>MeOH²</td>
<td>MeOH/HCl³</td>
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<tr>
<td>Naringenin</td>
<td>5.8 ± 0.3</td>
<td>5.4 ± 0.4</td>
<td>140 ± 10</td>
</tr>
<tr>
<td>Pinocembrin</td>
<td>ND</td>
<td>ND</td>
<td>180 ± 30³</td>
</tr>
<tr>
<td>Phloretin</td>
<td>7.1 ± 0.4</td>
<td>7.2 ± 0.3</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>2',4',6'-Trihydroxydihydrochalcone</td>
<td>5.2 ± 0.3</td>
<td>4.4 ± 0.1</td>
<td>200 ± 10</td>
</tr>
</tbody>
</table>

² The supernatant of the yeast culture was mixed with an equal volume of methanol before HPLC injection.
³ The supernatant of the yeast culture (5 ml) was freeze-dried and treated with a methanol-HCl solution.
⁴ The cell pellet (from 5 ml of yeast culture) was lyophilized and extracted with a methanol-HCl solution.
⁵ The cell pellet (from 5 ml of yeast culture) was lyophilized and extracted with a methanol-HCl solution.
⁶ P < 0.1.
⁷ ND, not detectable.
⁸ P < 0.05.

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.

Production of flavonoids in yeast pad1 knockout strain. It has been reported that the endogenous yeast enzyme phenylacetic acid decarboxylase (Pad1P, encoded by PAD1) is responsible for the decarboxylation of trans-cinnamoyl and p-coumaroyl 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-cinnamoyl acid production was 10-fold higher than that of p-coumaroyl 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

Production of flavonoids in yeast pad1 knockout strain. It has been reported that the endogenous yeast enzyme phenylacetic acid decarboxylase (Pad1P, encoded by PAD1) is responsible for the decarboxylation of trans-cinnamoyl and p-coumaroyl 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-cinnamoyl acid production was 10-fold higher than that of p-coumaroyl 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).
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 pad1I 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 pad1I knockout yeast suggested that Pad1P is not the only enzyme involved in the production of the dihydrochalcones in the pad1I 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 tetraketide 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 $\mu$M). However, the $V_{\text{max}}$ 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 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.

ACKNOWLEDGMENTS

We thank BioMarin Pharmaceutical Inc. (Novato, Calif.) for the phenylalanine ammonia lyase gene, C. Chapple (Biochemistry, Purdue University) for the 4-coumarate:CoA ligase gene, L. Beerthues (Institute of Pharmaceutical Biology, Germany) for the chalcone synthase gene, and N. Ho (LORRE, Purdue University) for the GAL10 promoter sequence, the XSS1 termination sequence, the pKS2a-Hyg plasmid, and S. cerevisiae AH22. We thank M. Sedlak for his helpful suggestions and technical assistance and G. Shaner for his help with revising the manuscript. We also thank D. Winski and D. Miles for their help with sample analysis.

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