Biosynthesis of 5-deoxyflavanones in microorganisms

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Flavanones are the common precursors of plant polyphenolic compounds collectively known as flavonoids. Leguminous plants have evolved a distinct class of flavanone molecules, known as 5-deoxyflavanones that play important roles in their symbiotic interactions. A four-step metabolic circuit was constructed in Escherichia coli with plant genes from heterologous origins: 4-coumarate:coenzyme A ligase from Petroselinum crispum, chalcone synthases (CHS) from Medicago sativa and Petunia x hybrida and chalcone reductase and chalcone isomerase from M. sativa. Evaluation of the different recombinant strains in shake flask experiments demonstrated that P. hybrida rather than M. sativa CHS resulted in the highest liquiritigenin production levels in glucose minimal medium, starting from precursor p-coumaric acid. Expression of the same recombinant pathway in Saccharomyces cerevisiae resulted in the accumulation of both 5-hydroxyflavanone and 5-deoxyflavanone, with the yields of the later lower than that achieved in E. coli. Other phenylpropanoid acid precursors, such as cinnamic acid and caffeic acid could also be metabolized through the recombinant pathway, yielding corresponding 5-deoxyflavanone compounds. The construction of such recombinant strains for 5-deoxyflavanone biosynthesis offers an alternative way to biochemically characterize flavonoid biosynthetic enzymes and promising production platforms for the biosynthesis of such high-value natural products.

Keywords: 5-Deoxyflavanones · Flavanones · Flavonoids · Liquiritigenin

1 Introduction

Flavonoid polyphenols are plant secondary metabolites that play important roles in plant biochemistry and physiology. They act as enzyme inhibitors, and are involved in UV protection, photosensitization and energy transfer, defense against infections etc. [1–4]. In recent years, flavonoids have been investigated extensively for their medicinal and nutraceutical properties [5]. For example, the flavanone liquiritigenin has been shown to exert cytoprotective effects during cadmium exposure [6] and to cause a significant reduction in serum cholesterol in rats [7]. Currently, the majority of these compounds are derived through plant extraction, plant cell cultures or through chemical synthesis. However, the health-promoting effects of flavonoids have stimulated research efforts towards the development of production platforms for their biosynthesis that employ well-characterized microbial hosts [8–20].

Biosynthesis of flavanones, the common precursors of the vast majority of flavonoids, starts with the phenylpropanoid biosynthetic pathway (Fig. 1). Cinnamic acid is converted to p-coumaric acid by a P450 monooxygenase, cinnamate 4-hydroxylase (not shown in Fig. 1). In the following step, p-coumaric acid is converted into 4-coumaroyl-CoA by 4-coumaroyl:CoA ligase (4CL). Next, chalcone synthase (CHS), the first committed step in flavonoid biosynthesis, catalyzes the sequential decarboxylative condensation of three acetate units from malonyl-CoA to 4-coumaroyl-CoA. This results in a linear phenylpropanoid tetraketide that forms 4,2',4',6'-tetrahydroxylchalcone via intramolecular cyclization and aroma-
tization [21]. In addition to CHS, leguminous plants have evolved a branched pathway that involves a monomeric polyketide reductase, chalcone reductase (CHR) [22]. This enzyme is believed to catalyze the stereospecific reduction of an intermediate of the multistep CHS reaction yielding chalcone and 4,2',4'-trihydroxychalcone (commonly referred to as 6'-deoxychalcone). The later product can then undergo various enzymatic modifications. Therefore, CHR offers an evolutionary advantage to leguminous plants, allowing them to synthesize unique polyphenolic molecules in response to interactions with other organisms such as symbiotic azotrophic bacteria, herbivores and plant pathogens [23]. Both 6'-deoxychalcones and tetrahydroxychalcones can then undergo isomerization, catalyzed by the enzyme chalcone isomerase (CHI) into their corresponding (2S)-flavanones [24]. CHIs are classified into two types: type I CHIs are found in non-legumes and isomerize only tetrahydroxychalcones; on the other hand, most of the CHIs of leguminous plants are type II enzymes and are active toward both 6'-deoxychalcones and tetrahydroxychalcones, yielding 5-deoxyflavanones and 5-hydroxyflavanones respectively [25, 26].

In the present work, we report the engineering of recombinant microbial systems for the in vivo functional analysis of metabolic enzymes involved in 5-deoxyflavanone biosynthesis. We initially characterized in vitro two CHI enzymes isolated from petunia and alfalfa and a CHR enzyme isolated from alfalfa by expressing them in E. coli. Co-expression in E. coli of 4CL-2 from parsley, CHR and CHI-1 from alfalfa and either CHS4-1 or CHS12-1

Figure 1. Flavanone biosynthesis in plants. C4H, cinnamate 4-hydroxylase.
(both from alfalfa) or CHS from petunia resulted in the biosynthesis of 5-deoxyflavanones with different efficiency. Introduction of the recombinant pathway in S. cerevisiae led to similar liquiritigenin production levels as the ones observed in E. coli. Physiological studies with different recombinant strains, optimization of liquiritigenin biosynthesis as well as biosynthesis of other 5-deoxyflavanones are also presented. The present work demonstrates the biosynthesis of 5-deoxyflavanones in microorganisms for the first time and provides an alternative way for studying interactions among their biosynthetic enzymes, as recently been demonstrated for other phenylpropanoid and flavonoid metabolic enzymes [16, 17].

2 Materials and methods

2.1 Strains and plasmids

E. coli TOP10F' (Invitrogen, Carlsbad, USA) and DH5α were used for DNA manipulations, while E. coli BL21 Star (Novagen) and S. cerevisiae INVSC1 (Invitrogen) were used for shake-flask experiments. Plasmids pYES2.1/V5-His-TOPO (Invitrogen), YEplac181 (ATCC no. 87588), pETDuet-1, pCDFDuet-1 and pRSFDuet-1 and pCO-LADuet-1 (Novagen) were used for cloning purposes. All strains and plasmids are listed in Table 1.

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a) Amp, ampicillin; Sm, streptomycin; Kan, kanamycin.

2.2 Chemicals

p-Coumaric acid was purchased from MP Biomedicals Inc (USA), naringenin was purchased from Sigma-Aldrich (St. Louis, USA) and liquiritigenin, isoliquiritigenin, pinocembrin, 7-hydroxyflavanone, 2',4'-dihydroxychalcone, eriodictyol and butein were purchased from Indofine (New Jersey, USA). Butin was purchase from Apin Chemicals (Abingdon, UK). Tetrahydroxychalcone was chemically synthesized from naringenin as previously described [27]. Luria Broth (LB) rich medium was used for E. coli propagation, while M9 minimal medium (1 x M9 salts, 0.2% glucose, 6 nM thiamine, 1 μM MgSO_4_2_, 0.1 μM CaCl_2_) and Terri Broth (TB) rich medium were used for E. coli shake flask experiments. Yeast cell cultures were performed in SC-Leu and SC-Leu-Ura minimal media prepared as previously described [12].

2.3 DNA manipulations

All DNA manipulations were performed according to standard procedures [28]. Restriction enzymes were purchased from New England Biolabs or Promega. T4 DNA ligase was purchased from Invitrogen. RT-PCR reactions were carried out by using Superscript One-step w/ platinum Taq kit (Invitrogen). PCR reactions were performed using Bioline’s Accuzyme. MsCHS4-1 (accession no. 1252 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
U01018) and MsCHS12-1 (accession no. U01021) cDNA were kind gifts from Dr. Ann Hirsch (Department of Molecular, Cell and Developmental Biology, UCLA) [29]. Isolation of Pc4cL-2 cDNA from Petroselinum crispum (accession no. X13325), CHI-A and chs from Petunia x hybrida corolla (accession nos. M91079, L02901 and X82366, respectively) from Medicago sativa young leaves were all amplified by RT-PCR. All primers used are listed in Table 2. In all cases, the absence of mutations was verified by direct nucleotide sequencing.

2.4 Plasmid construction and gene cloning

Plasmid pCDF-PC4CL2 was constructed by cloning Pc4cL-2 cDNA between EcoRV and KpnI in vector pCDF-Duet-1. Plasmid pCDF-PC4CL2-MsCHR was created by cloning CHR cDNA between EcoRI and SalI in vector pCDF-PC4CL2. CHR cDNA was cloned in vector pRSF-Duet-1 using EcoRI and SalI, yielding plasmid pRSF-MsCHR. MsCHI-1 cDNA was cloned in pETDuet-1 between EcoRV and KpnI, yielding plasmid pET-MsCHI. In the next step, chs, MsCHS4-1, MsCHS12-1 and a M. sativa CHS-like cDNA isolated in our laboratory were cloned in pET-MsCHI between EcoRI and SalI, creating the respective plasmids pET-MsCHI-PhCHS, pET-MsCHI-MsCHS4-1, pET-MsCHI-MsCHS12-1 and pET-MsCHI-MsCHS. P. hybrida chs cDNA was cloned in pETDuet-1 between EcoRI and SalI, yielding plasmid pET-PhCHS.

For constructing the 5′-deoxyflavanone recombinant pathway in S. cerevisiae, MsCHI-1 cDNA was cloned by T/A cloning into vector pYES2.1/V5-His-TOPO (Invitrogen), yielding plasmid pYES2.1-MsCHI-1. In the next step, the CHI-1 cDNA was amplified together with the GAL1 promoter and cloned in plasmid Ycc4c-181 [12] by restriction digest with KpnI and EcoRI yielding plasmid Ycc4mc-181. CHR cDNA was subcloned in vector pYES2.1/V5-His-TOPO (Invitrogen) by T/A cloning, yielding plasmid pYES2.1-MsCHR. All primers used are listed in Table 2.

Yeast transformation was carried out using the lithium acetate method [31].

2.5 In vitro CHI and CHR assays

E. coli BL21Star recombinant strains harboring plasmids pET-PhCHI, pET-MsCHI or pETDuet-1 (control) were pre-inoculated in 3 mL LB rich medium supplemented with 50 μg/mL ampicillin and were left to grow at 37°C overnight with shaking. The following day, 1 mL pre-inoculum was added to 50 mL fresh LB medium (also containing the same concentration of ampicillin) and the culture was left to grow at 37°C with shaking until OD₆₀₀
reached approximately 0.6. At that point, isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM and the culture was left to grow at 30°C for 5 h. Cells were harvested by centrifugation, washed twice with washing buffer (0.9% NaCl) and resuspended in lysis buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.4). Cell disruption was performed using glass beads and the enzyme crude extract was collected by centrifugation at 4000 rpm for 10 min. Total protein was estimated using the BCA protein assay (Pierce Chemicals).

For measuring chalcone isomerase activity, 2 μL isoliquiritigenin stock solution (2 μg/μL) was added to 200 μL crude extract and the mixture was incubated at 30°C for 5 min. After reaction termination by acidification, the reaction was twice extracted with equal volume of ethyl acetate; the organic layer was combined together and subjected to speed vacuum. The dry powder was dissolved with 10 μL DMSO and 40 μL water and was subjected to HPLC analysis. For the study of CHI kinetic properties, ranges of isoliquiritigenin substrate concentrations between 78 and 27 μM were employed for determining $K_m$ value. The apparent Michaelis-Menten constant for CHI was obtained from a Lineweaver-Burk plot of the resulting data.

The standard mixture for the CHR enzymatic assay was prepared by mixing 1 μL 250 mM p-coumaric acid, 20 μL 500 mM ATP, 20 μL 500 mM magnesium sulfate, 30 μL 50 mM coenzyme-A, 30 μL 10 mM malonyl-CoA and 2 mg NADPH powder with 200 μL 4CL crude extract (1.0–1.5 μg/μL total protein concentration), 200 μL of CHS crude extract (1.0–1.5 μg/μL total protein concentration) and 200 μL CHR crude extract (1.0–1.5 μg/μL total protein concentration). After 3 h at 30°C the reaction mixture was stopped by acidification, twice extracted with equal volume of ethyl acetate, dried under vacuum and subjected to HPLC analysis.

To study the substrate specificity of CHR, a different assay reaction was setup as follows: 5 μL tetrahydroxycalcone stock solution (2 μg/μL) were mixed with 2 mg NADPH and 495 μL CHR crude extract (1.0–1.5 μg/μL). The mixture was incubated at 30°C for 30 min after which flavonoid compounds were extracted twice with equal volume of ethyl acetate, dried and analyzed by HPLC.

2.6 Shake flask experiments

Five E. coli BL21Star recombinant strains harboring either of the plasmids pET-PhCHS, pET-MsCHI-PhCHS, pET-MsCHI-MsCHS4-1, pET-MsCHI-MsCHS12-1 or pET-MsCHI-MsCHS together with plasmids pCDF-PC4CL2 and pRSF-MsCHR were used for shake flask experiments in M9 minimal medium, LB and TB rich media. Each of the recombinant strains was pre-inoculated into 3 mL M9 or LB or TB cultures supplemented with 30 μg/mL kanamycin, 50 μg/mL streptomycin and 50 μg/mL ampicillin and incubated at 37°C with vigorous shaking overnight. The next day, 1 mL pre-inoculum culture was inoculated into 50 mL of the corresponding media and the culture was left to grow at 37°C with vigorous shaking at 300 rpm to an OD$_{600}$ of approximately 0.6. At that point IPTG was added to the culture to a final concentration of 1 mM. To avoid the formation of inclusion bodies, after the addition of the inducer, the culture was incubated at 30°C with vigorous shaking for 3 h. The precursor phenylpropanoid acid was then added and the culture was left to grow at 30°C for 65 h.

For the yeast shake flask experiments, a single colony of the yeast recombinant strain carrying plasmids Ycc4mc-181 and pYES2.1-MsCHR was inoculated into 1 mL SC-Leu-Ura minimal medium containing 2% glucose and was left to grow overnight at 30°C with shaking. The next day, a 10-mL culture containing induction medium (SC-Leu-Ura supplemented with 2% galactose) was inoculated from the pre-inoculum culture (washed twice with water and resuspended in induction medium) to an initial OD$_{600}$ of 0.4 and was left to grow at 30°C with vigorous shaking for 6 h. At that time, phenylpropanoid acid precursor was added to the culture to a final concentration of 1.5 mM and the culture was left to grow at 30°C for another 7 days.

2.7 Flavonoid extraction

After completion of the shake flask experiments, flavonoids were extracted directly from 100 μL culture with 150 μL ethyl acetate for 30 s at room temperature with vigorous shaking. After centrifugation at 14 000 rpm for 2 min, 100 μL of the organic layer was collected and evaporated to dryness by speed vacuum. The resulting powder was dissolved in 10 μL DMSO and 40 μL water; 30 μL of this was injected into HPLC for analysis and quantification purpose.

2.8 HPLC analysis

Flavonoids were analyzed by HPLC using an Agilent 1100 series instrument and a reversed phase ZORBAX SB-C18 column (4.6 × 150mm) maintained at 25°C. The compounds produced were separated by elution with an acetonitrile/water gradient, at a flow rate of 1.0 mL/min. The HPLC conditions were as follows: 10–40% acetonitrile for 10 min, 40–60% acetonitrile for 5 min, and 60–10% acetonitrile for 10 min. The retention times under these HPLC conditions for the standard authentic samples are presented in Table 3. Flavanones were identified by retention time and UV absorption spectra plotted by a diode array detector and detected and quantified by monitoring absorbance at 290 nm, while the corresponding chalcones were identified and quantified by monitoring absorbance at 367 nm. The quantitative calibration curves were obtained with standard flavanone and chalcone solutions with various concentrations.
### 3 Results

#### 3.1 CHI activity

Since a number of enzymes used in the present study have been functionally expressed in the past by our group for the biochemical synthesis of flavonoid molecules in *E. coli* and *S. cerevisiae* [11, 12, 15, 30, 32], we investigated the functional expression in *E. coli* of two new enzymes required for 5-deoxyflavanone biosynthesis, namely a type II CHI and a CHR both isolated from alfalfa.

CHI (EC 5.5.1.6) catalyzes the stereospecific isomerization of tetrahydroxychalcone (2',4,4',6'-tetrahydroxychalcone) and 6'-deoxychalcones to (2S)-naringenin (4',5,7-trihydroxyflavanone) and (2S)-5-deoxyflavanones (4',7-dihydroxyflavanone), respectively. Although naringenin chalcone isomerizes spontaneously to a racemic mixture of (2R)- and (2S)-flavanones, only (2S)-flavanones can be further metabolized by flavonoid biosynthetic enzymes. In that respect, CHI guarantees the efficient formation of biologically active (2S)-isomers.

We tested the *in vitro* enzyme activity of a type I CHI (*P. hybrida*) and a type II CHI (*M. sativa*) expressed in *E. coli* towards 6'-deoxychalcones by *in vitro* assay and kinetic study. As expected, the petunia CHI showed no activity towards isoliquiritigenin (Fig. 2B). On the other hand, the *M. sativa* CHI-1 converted approximately 95% of isoliquiritigenin into liquiritigenin in less than 10 min (Fig. 2D) with a *K_m* value equal to 517 ± 19 μM. Therefore, *M. sativa* CHI-1 was further used for 5-deoxyflavanone biosynthesis in both *E. coli* and *S. cerevisiae*.

#### 3.2 CHR activity

CHR is the key enzyme that controls the production ratio of 5-deoxyflavonoids and 5-hydroxyflavonoids in plants [33, 34]. Several CHR cDNAs have been cloned from soybean (*Glycine max*) [35], from kudzubean (*Pueraria lobata*) [36], alfalfa (*Medicago sativa*) [33, 34] and licorice (*Glycyrrhiza echinata*) [36].

In the present study, CHR was amplified from young leaves of alfalfa and was cloned into vector pRSFDuet-1. To confirm the functional expression of the recombinant protein, an assay system was devised that included 4CL, which catalyzes the formation of 4-coumaroyl-CoA from coumaric acid and coenzyme-A, and CHS and CHR, which catalyze the formation of chalcones from 4-coumaroyl CoA. As shown in Fig. 3B, both 4,2',4',6'-tetrahydroxychalcone and 4,2',4'-trihydroxychalcone were formed in the assay reaction. The formation of naringenin was also detected as a product of the spontaneous chemical isomerization of 4,2',4',6'-tetrahydroxychalcone. No liquiritigenin (Fig. 3B) was detected in the assay system, probably because 4,2',4'-trihydroxychalcone is more stable than 4,2',4',6'-tetrahydroxychalcone [26]. As a control (Fig. 3C), when the CHR crude extract was replaced by crude extract from *E. coli* carrying blank vector pRSFDuet-1, no 4,2',4'-trihydroxychalcone was detected. As another control (Fig. 3D), when the CHS crude extract was replaced by crude extract from *E. coli* carrying blank vector pETDuet-1, no chalcones could be detected.

CHR has been less studied biochemically compared to the other enzymes in the flavanone biosynthetic pathway [33], and its substrate specificity is still under investigation [23, 35]. Theoretically, CHR should catalyze the reduction of the carbonyl group of one or more intermediates from the chain elongation steps of CHS, which are mainly linear di, tri, and tetra-ketide CoAs [23]. However,
according to Oguro’s recent findings \[37\], the linear di and triketide-CoA intermediates have been excluded from consideration as substrate candidates since the CHS cannot perform further polyketide chain elongation on the reduced diketide-CoA intermediate. With the recent elucidation of the CHR structure from \textit{M. sativa}, the non-aromatized coumaryl-trione intermediate was rationalized as a potential substrate \[23\]. Although the prevailing assumption is that 4,2’,4’,6’-tetrahydroxychalcone cannot be accepted as a substrate by CHR, we tested this hypothesis by following the biosynthesis of isoliquiritigenin using HPLC starting from the unstable substrate 4,2’,4’,6’-tetrahydroxychalcone, which was chemically synthesized. Three different amounts of tetrahydroxychalcone were tested (1.0, 5.0 and 10.0 μg) by incubating the assay mixture at 30°C for 30 min and extracting flavonoid compounds twice with equal volume of ethyl acetate. Isoliquiritigenin biosynthesis was verified by HPLC analysis and by matching the retention times and UV absorbance profiles of the extracted flavonoid compounds with authentic compounds (Figs. 4A and B). No isoliquiritigenin formation was identified in a control experiment using protein crude extract from a BL21Star strain harboring empty vector (Fig. 4C). The presence of a naringenin peak in both experiments was attributed to the spontaneous isomerization of 4,2’,4’,6’-tetrahydroxychalcone. The control experiment using naringenin as substrate (Fig. 4D) showed that CHR cannot accept it as substrate. It is important to note that due to 4,2’,4’,6’-tetrahydroxychalcone’s instability, enzyme activity measurements were based on product formation rather than substrate consumption; as such, a $K_m$ value for CHR was not obtained.

### 3.3 Engineering the liquiritigenin biosynthetic pathway in \textit{E. coli}

Having demonstrated the functional expression of alfalfa CHR and CHI-1 in \textit{E. coli}, we proceeded with the reconstruction of the liquiritigenin biosynthetic pathway in this microorganism. Biosynthesis of liquiritigenin from precursor phenylpropanoid acids involves a four-enzyme pathway consisting of 4CL, CHS, CHR and CHI. The recombinant network tested included either of two CHS enzymes (CHS4-1 and CHS12-1) previously isolated by Mc-
Khann and Hirsch from *M. sativa* [29] but not yet characterized biochemically or a CHS enzyme isolated from *P. hybrida* petals.

The recombinant pathway was assembled using vectors pCDFDuet-1, pRSFDuet-1 and pETDuet-1 that can co-replicate in *E. coli* and carry three different antibiotic-resistance genes for selection purposes. *Pc4cL-2* was cloned into the low-copy-number vector pCDFDuet-1, *CHR* was cloned into high-copy-number vector pRSFDuet-1 and the medium copy-number vector pETDuet-1 was employed to carry any of the four different chs genes together with the *MsCHI-1* cDNA from *M. sativa*. All genes were cloned individually under the strong T7 promoter present in all three vectors.

### 3.4 Flavanone production by recombinant *E. coli* strains

*E. coli* recombinant strains expressing a recombinant pathway consisting of 4CL, CHS and CHR, in the presence or absence of type I or type II CHIs were tested for the production of liquiritigenin in M9 minimal medium as well as LB and TB rich media. At an OD<sub>600</sub> of approximately 0.6, 1 mM IPTG was added to the cell cultures to induce the protein expression. After the addition of IPTG, the cell cultures were grown at 30°C for 3 h to avoid the formation of recombinant protein inclusion bodies. Subsequently, *p*-coumaric acid was added to the cell cultures at a final concentration of 0.5 mM. Flavonoid compounds were extracted with ethyl acetate after 65 h fermentation. Isoliquiritigenin, liquiritigenin and naringenin were detected in all cell cultures and their end-point concentrations are presented in Table 4.

In a control experiment, where no CHI was present in the pathway, *E. coli* recombinant strain expressing parsley 4CL-2, alfalfa CHR and petunia CHS resulted in the production of 3.6 mg/L isoliquiritigenin, 3.7 mg/L liquiritigenin and 20.6 mg/L naringenin in M9 minimal medium. A slight increase in the production of flavanone compounds was observed in TB rich media, with 8.7% and 22% increase in the concentration of naringenin and liquiritigenin, respectively. However, much lower production was observed in LB rich medium. No 4',2',4',6'-tetrahydroxychalcone was detected in any culture media, most likely due to its isomerization to naringenin during fermentation or sample preparation. Even though isoliquiritigenin is more stable than naringenin chalcone, spontaneous isomerization still occurred in the culture medium during the fermentation period or sample preparation that lasted more than 2 days. Such isomerization was not observed in the *in vitro* assay, simply because the assay’s time duration is a few minutes as opposed to days required for the fermentation and compound extraction.

When alfalfa CHI-1 was added to the biosynthetic pathway, an increase in the amount of liquiritigenin and naringenin was observed in all culture media (M9, LB and TB). The best production of flavanone compounds was observed in M9 minimal medium with 105% increase in the amount of liquiritigenin and 18% increase in the amount of naringenin compared with the previous control experiment (no CHI). The dramatic decrease (64%) in the production of intermediate isoliquiritigenin was mainly because of the introduction of alfalfa CHI into the cells, which converted most of isoliquiritigenin into liquiritigenin. Similarly, the increase in the amount of flavanone

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compounds and decrease in the amount intermediate isoliquiritigenin was observed in both LB and TB with the presence of CHI. An interesting finding was that the introduction of alfalfa CHI led to an approximately 20% increase in the total amount of flavonoids produced compared to the previous control experiment (absence of CHI).

When the petunia CHS was replaced by alfalfa CHS4-1 or CHS12-1, biosynthesis of naringenin and liquiritigenin was observed, verifying that these enzymes are part of the flavonoid biosynthetic pathway in alfalfa (Table 4). Contrary to our expectation, however, the overall liquiritigenin and naringenin production decreased when these two enzymes were employed instead of the petunia CHS. More specifically, isoliquiritigenin, liquiritigenin and naringenin production dropped by 7.7%, 7.9% and 9.4%, respectively, in M9 minimal medium, 9.7%, 9.1% and 6.9% in LB medium, and 16.9%, 20.8% and 8.4% in TB medium. When CHS12-1 was employed, isoliquiritigenin, liquiritigenin and naringenin production dropped by 23%, 18% and 21% in M9 minimal medium, 39%, 21% and 23% in LB medium, and 37%, 32% and 24% in TB medium. This result can be considered a direct reflection of the in vivo enzymatic activities of different CHS heterologously expressed in E. coli.

Overall, the production profiles of flavanone compounds and intermediates with different recombinant strains are similar to each other in different culture media (M9, LB and TB). Better production was always observed in M9 and TB cultures. Without the presence of CHI in the biosynthetic pathway, a small increase in the production of flavanone compounds in TB medium was observed compared with M9 medium. However, the presence of CHI in the biosynthetic pathway reversed such phenomena. From all the studies related to combinations of different heterologous genes and culture media, the recombinant E. coli expressing parsley 4CL-2, alfalfa CHR, petunia CHS and alfalfa CHI fermented in M9 minimal medium presented the highest production with 7.6 mg/L liquiritigenin and 24.4 mg/L naringenin.

### 3.5 Optimization of 5-deoxyflavanone production in E. coli

As previously described, the four flavanone biosynthetic genes were cloned in three compatible vectors with different selective markers. To further optimize flavanone biosynthesis, the CHR gene was removed from pRSF-Duet-1 by restriction digest using EcoRI and SalI and introduced in vector pCDF-PC4CL2, under the T7 promoter, thus removing one vector from the recombinant strain and the need for the kanamycin selection antibiotic. The resulting strain was tested by fermentation in M9 minimal medium supplemented with 0.1, 0.25, 0.5, and 1.0 mM p-coumaric acid. As demonstrated in Table 5, naringenin production increased by 38% (from 24.4 to 33.7 mg/L) and liquiritigenin production by 122% (from 7.6 to 16.9 mg/L) when the same concentration of precursor phenylpropanoid acid (0.5 mM coundaric acid) was present in the culture as described above.

#### 3.6 Reconstruction of the liquiritigenin biosynthetic pathway in S. cerevisiae

We also tested the ability of yeast to produce liquiritigenin, by reconstructing the biosynthetic network consisting of parsley 4CL-2, petunia CHS (the enzyme that gave the best production in E. coli), and alfalfa CHI-1 and CHR. The first three enzymes were cloned in the high-copy-number vector YEplac181 under the control of galactose induced GAL1 yeast promoter, as previously described [12], while CHR was cloned in vector pYES2.1 also under the GAL1 promoter. Co-replicaton of the two plasmids was based on different selection markers (Leu– for YEplac181 and Ura– for pYES2.1).

The yeast recombinant strain carrying Ycc4m181 and pYES2.1-MsCHR was employed for liquiritigenin production in Sc-Leu-Ura minimal medium supplemented with p-coumaric acid at final concentration of 1.5 mM. As shown in Table 6, the recombinant yeast cells harboring the hybrid pathway exhibited significantly higher overall flavanone productivity compared with recombinant E. coli cells. More specifically, naringenin production increased by 246% reaching 118.9 mg/L compared with recombinant E. coli cells: however: the liquiritigenin production dropped by 20% reaching 13.5 mg/L. As in the case of E. coli, no accumulation of tetrahydroxychalcone was observed in the fermentation broth and around 1.25 mM coumaric acid was consumed from the medium in 7 days, thus providing an overall conversion yield of 40.2%.

<table>
<thead>
<tr>
<th>Substrate consumed (mM)</th>
<th>Total products (mg/L)</th>
<th>Conversion ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM</td>
<td>7.5 ± 0.5</td>
<td>0.84 ± 0.17</td>
</tr>
<tr>
<td>0.25 mM</td>
<td>19.7 ± 2.5</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>33.7 ± 3.7</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>34.4 ± 4.4</td>
<td>3.5 ± 0.8</td>
</tr>
</tbody>
</table>

*Table 5.* The production of isoliquiritigenin, liquiritigenin and naringenin and precursor phenylpropanoid acid conversion ratio by recombinant E. coli BL21star cells harboring parsley 4CL-2, petunia CHS, alfalfa CHR and CHI in two vectors4.

a) Shake flask experiments were performed in M9 minimal medium for 65 h.
3.7 Exploring the biosynthesis of other 5-deoxyflavanones from recombinant microorganisms

Both the recombinant *E. coli* BL21Star strain and recombinant *S. cerevisiae* harboring the pathway were tested for the biosynthesis of other 5-deoxyflavanones besides liquiritigenin. For that purpose, cinnamic acid and caffeic acid were tested as precursor metabolites.

First, we determined the optimal feeding strategy for the two precursor phenylpropanoid acids. For that purpose, cinnamic acid was added in M9 minimal medium (*E. coli*) or SC-minimal medium (yeast) at a final concentration of 0.5 mM, and its consumption was monitored by sampling the fermentation broth every 24 h. In the case of yeast, cinnamic acid disappeared in the first 24 h and an additional amount was added during the course of the fermentation to achieve the highest production level. In the case of *E. coli*, however, addition of 0.5 mM cinnamic acid at the beginning of the fermentation proved sufficient and residual amount could still be detected at the end of the 65-h fermentation. Similarly, addition of 0.5 mM caffeic acid in both yeast and *E. coli* recombinant cultures proved to be sufficient for achieving the highest production and residual amount of the precursor could be detected after the fermentation termination (Fig. 5). Based on these results, in the case of cinnamic acid yeast fermentation, the precursor phenylpropanoid acid was added in six equal doses of 0.5 mmol every 24 h, during a 7-day fermentation period. In the case of cinnamic acid *E. coli* fermentation and both yeast and *E. coli* caffeic acid fermentations, the precursor phenylpropanoid acids were added once to final concentration of 0.5 mM at the beginning of the fermentation experiments.

Both cinnamic acid and caffeic acid were metabolized by the recombinant strains. More specifically, in the case of *E. coli*, 28.5 mg/L pinocembrin, 1.9 mg/L 7-hydroxyflavanone and 1.0 mg/L 2',4'-dihydroxychalcone accumulated in the medium starting from cinnamic acid, thus resulting in a cinnamic acid to 7-hydroxyflavanone conversion ratio of 30.5%. When caffeic acid was utilized as a precursor, 5.2 mg/L eriodictyol, 4.2 mg/L butin and 0.5 mg/L butein were produced, resulting in a caffeic acid to butin conversion ratio of 32.7%. With *S. cerevisiae* as host, the overall flavanone production increased dramatically: pinocembrin reached 57.2 mg/L and eriodictyol reached 7.9 mg/L, respectively. However, the production of corresponding 6'-deoxychalcone and 5-deoxyflavanone decreased (1.0 mg/L 2',4'-dihydroxychalcone and 1.9 mg/L 7-hydroxyflavanone: 0.5 mg/L butein and 4.2 mg/L butin), as previously observed for the case of liquiritigenin. As a result, the conversion ratio dropped to 11.0% for cinnamic acid and 24.2% for caffeic acid. The biotransformation results are summarized in Tables 7 and 8.

4 Discussion

We present the construction and optimization of *E. coli* and yeast recombinant strains that allow the biosynthesis of natural 5-deoxyflavanones from precursor phenylpropanoid acids. This was achieved by expressing a type II CHI isolated from alfalfa in both microorganisms, as type I CHI that has previously been described in flavonoid metabolic engineering efforts [15, 32] cannot accept 6'-deoxychalcones as substrates. In addition, the functional expression of alfalfa CHR is also required, which was reported only very recently in *E. coli* and while our work was in progress [23].

Microbial in vivo systems, such as the one presented here, can provide several future opportunities for studying the biochemical properties of flavonoid biosynthetic enzymes. Recently, Ralston et al. [16] and Ro and Douglas [17] demonstrated the use of yeast recombinant strains in further elucidating the role of protein-protein interactions in controlling metabolic flux through the phenylpropanoid and isoflavone biosynthetic pathways, respectively. In addition, with the large accumulation of genomic information, such recombinant pathways can be useful for the biochemical characterization of flavonoid biosynthetic genes. We present such examples here, by testing three CHS-like enzymes among the several that have been identified in alfalfa [29, 38] and the more than 650 CHS-like sequences that have been isolated from plant and microbial species [21] for their ability to catalyze liquiritigenin biosynthesis from p-coumaric acid. Two of them, CHS4-1 and CHS12-1 demonstrated catalytic activity and resulted in similar amounts of liquiritigenin production. Surprisingly, the presence of a CHS enzyme isolated from petunia (a non-leguminous plant) resulted in the highest

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Naringenin (mg/L)</th>
<th>LQN (mg/L)</th>
<th>ILQN (mg/L)</th>
<th>Substrate consumed (mM)</th>
<th>Total products (mM)</th>
<th>Conversion ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumaric acid 1.5 mM</td>
<td>118.9 ± 5.6</td>
<td>13.5 ± 1.2</td>
<td>3.2 ± 0.3</td>
<td>1.25</td>
<td>0.503</td>
<td>40.2%</td>
</tr>
</tbody>
</table>
accumulation of liquiritigenin in recombinant *E. coli* when compared with the two CHS enzymes from *M. sativa*. It therefore appears that either CHR-CHS complexes, speculated to exist in leguminous plants [21], have no effect on carbon fluxes or that such complexes can form irrespective of whether CHS and CHR are derived from heterologous plant sources. It is also important to note that the presented data underline that bioprospecting of genetic elements from various plant sources is an important strategy for developing optimal biocatalysts, at least for

**Figure 5.** Product formation and precursor phenylpropanoid acid consumption profile of recombinant *E. coli* and yeast strains. Diamond: phenylpropanoid acid (cinnamic acid, *p*-coumaric acid, caffeic acid); square: corresponding 5-hydroxylflavonone (pinocembrin, naringenin and eriodictyol); triangle: corresponding 5-deoxyflavonone (7-hydroxyflavanone, liquiritigenin and butin); star: corresponding 6'-deoxychalcone (2',4'-dihydroxychalcone, isoliquiritigenin, butein). (A) Recombinant *E. coli* fermentation with 0.5 mM cinnamic acid; (B) recombinant *E. coli* fermentation with 0.5 mM *p*-coumaric acid; (C) recombinant *E. coli* fermentation with 0.5 mM caffeic acid; (D) recombinant *S. cerevisiae* fermentation with 3.0 mM (total) cinnamic acid; (E) recombinant *S. cerevisiae* fermentation with 1.5 mM *p*-coumaric acid; (F) recombinant *S. cerevisiae* fermentation with 0.5 mM caffeic acid.

**Table 7.** The production of 5-deoxyflavonone, flavanone, 6'-deoxychalcone and precursor phenylpropanoid acid conversion ratio by recombinant *E. coli* BL21 star cells harboring parsley 4CL-2, petunia CHS, alfalfa CHR and CHI in two vectors

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Products</th>
<th>Substrate consumed (mM)</th>
<th>Total products (mM)</th>
<th>Conversion ration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamic acid 0.5 mM</td>
<td>Pinocembrin (mg/L)</td>
<td>0.4</td>
<td>0.122</td>
<td>30.5%</td>
</tr>
<tr>
<td></td>
<td>HF (mg/L)</td>
<td>28.5 ± 2.3</td>
<td>1.9 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>DHc (mg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeic acid 0.5 mM</td>
<td>Eriodictyol (mg/L)</td>
<td>0.11</td>
<td>0.036</td>
<td>32.7%</td>
</tr>
<tr>
<td></td>
<td>Butin (mg/L)</td>
<td>5.2 ± 0.6</td>
<td>4.2 ± 0.3</td>
<td>0.5 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Butein (mg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Shake flask experiments were performed in M9 minimal medium for 65 h. HF, 7-hydroxylflavonone; DHc, 2',4'-dihydroxychalcone.
Table 8. The production of 5-deoxyflavanone, flavanone, 6’-deoxychalcone and precursor phenylpropanoid acid conversion ratio by recombinant 
*S. cerevisiae* cells harboring parsley 4CL-2, petunia CHS, alfalfa CHR and CHI

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Products</th>
<th>Substrate consumed (mM)</th>
<th>Total products (mM)</th>
<th>Conversion ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamic acid 3.0 mM</td>
<td>Pinocembrin (mg/L) 57.2 ± 4.8</td>
<td>HF (mg/L) 0.9 ± 0.03</td>
<td>DHC (mg/L) 0.5 ± 0.01</td>
<td>2.1 0.23 11.0%</td>
</tr>
<tr>
<td>Caffeic acid 0.5 mM</td>
<td>Eriodictyol (mg/L) 7.9 ± 0.5</td>
<td>Butin (mg/L) 2.5 ± 0.4</td>
<td>Butein (mg/L) 0.2 ± 0.02</td>
<td>0.19 0.037 19.5%</td>
</tr>
</tbody>
</table>

a) Shake flask experiments were performed in SC-Leu-U minimal medium for 7 days.

plant secondary metabolites. We plan to expand such efforts either for the biosynthesis optimization of flavonoid molecules that have recently been synthesized in our laboratory [11, 15, 18, 19, 32] or for new molecules currently under investigation.

The constructed in vivo systems can also present potentially competitive alternatives to current methods of flavonoid biosynthesis that in many cases involve plant cell cultures [39, 40] or plant extractions. Contrary to our recent finding on 5-hydroxyflavanone biosynthesis [12], yeast recombinant strains resulted in less liquiritigenin biosynthesis than from *E. coli* recombinant strains, while the amount of naringenin produced was approximately 3.6 times higher in yeast than in *E. coli*. Since both naringenin and liquiritigenin are synthesized through the same set of enzymes except for CHR, it is possible that CHR is the enzyme that exercises the highest metabolic flux control in the constructed metabolic network. This could be the result of poor expression, low biochemical activity, biochemical control, reduced precursor availability (NADPH) or a combination of these.

Finally, the constructed recombinant strains were also able to metabolize other phenylpropanoid acid precursors, such as cinnamic acid and caffeic acid. The corresponding 5-hydroxyflavanone production was higher in yeast cultures than in *E. coli*, while 5-deoxyflavanone and intermediate 6’-deoxychalcone was lower, a result that was consistent with our previous findings using cuminaraic acid. With the demonstrated substrate flexibility of the constructed biosynthetic networks, the biosynthesis of several other natural or non-natural 5-deoxyflavanones, as previously described for flavanones and dihydroflavonols [19] could be possible and is currently under investigation.

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