Characterization of dihydroflavonol 4-reductases for recombinant plant pigment biosynthesis applications

EFFENDI LEONARD1, YAJUN YAN1, JOSEPH CHEMLER1, ULRICH MATERN2, STEFAN MARTENS2, & MATTHEOS A.G. KOFFAS1

1Department of Chemical and Biological Engineering, the State University of New York at Buffalo, Buffalo, NY 14260, USA and 2Institut für Pharmazeutische Biologie, Philipps-Universität Marburg, Deutschhausstrasse 17A, D-35037, Marburg, Germany

Abstract
Anthocyanins are colorful plant pigments with promising applications as pharmaceuticals and colorants. In order to engineer efficient pigment biosynthesis in Escherichia coli, the activities of various dihydroflavonol 4-reductases (DFRs) were characterized for the three primary dihydroflavonol substrates. The biochemical assays demonstrated variable DFR activities for dihydroflavonol with one B-ring hydroxyl group, the precursor of pelargonidin derivatives. In contrast, dihydroflavonols with two and three B-ring hydroxylation were metabolized with comparable efficiency. Furthermore, the catalysis of DFR for the secondary substrates, flavanones, also depended on the number of B-ring hydroxyl groups. Engineering the expression of the DFR clones together with plant-specific 4-coumaroyl:CoA ligase, chalcone synthase, chalcone isomerase, and flavanone 3-hydroxylase in E. coli resulted in the synthesis of pelargonidin at various levels, from p-coumaric acids. The identification of a robust DFR from this study can also be used for engineering recombinant synthesis of other bioactive flavonoids, such as flavan-3-ols.

Keywords: Substrate specificity, substrate flexibility, dihydroflavonol 4-reductase, heterologous anthocyanidin biosynthesis, metabolic engineering, Escherichia coli

Introduction
More than half of the drugs currently in clinical use are either natural products or natural product derivatives (Paterson & Anderson 2005). Despite their great potential, pharmaceutical companies have yet to fully endorse these compounds, citing the lack of availability from their natural hosts as one of the bottlenecks for large-scale production. Often, chemical synthesis of natural products results in long developmental times and low yields. Similarly, plant cell cultures usually require long culturing times and result in low productivity (Roberts & Shuler 1997). The rapid advancement of metabolic engineering has allowed the engineering of easy to culture microbes, such as Escherichia coli, to serve as a production platform for plant secondary metabolites (Leonard et al. 2007). To this end, such biotechnological approaches can offer a competitive alternative to traditional technologies.

The anthocyanin family is one of the major plant secondary metabolite constituents that contribute to plant pigmentation. Today, anthocyanins have found applications as food colorants. Moreover, an increasing number of studies have demonstrated that anthocyanins exhibit anti-cancer and anti-obesity properties (Hagiwara et al. 2001; Hou et al. 2004; Tsuda et al. 2006). In plants, anthocyanin biosynthesis starts from the conversion of phenylpropanoic acid to CoA ester by the action of 4-coumaroyl:CoA ligase (4CL). Subsequently, chalcone synthase (CHS) condenses one CoA ester molecule with three malonyl-CoA moieties to form chalcone. Next, chalcone isomerase (CHI) catalyzes the isomerization of chalcone to form flavanone, the common precursor of many flavonoids. The first
committed step in anthocyanin biosynthesis is the conversion of flavanone into dihydroflavonol by flavanone 3-hydroxylase (FHT). Dihydroflavonol 4-reductase (DFR) then catalyzes the stereospecific reduction of (2R,3R)-dihydroflavonol to the respective colorless (2R,3S,4S)-leucoanthocyanidin. Following this reaction, anthocyanidin synthase (ANS) oxidizes leucoanthocyanidin into the anthocyanidin pseudobase. Transport to the acidic environment of the plant vacuoles allows the formation of the flavylium ion of anthocyanidin, creating colourful pigments, which are often further glycosylated to the more stable anthocyanins (Winkel-Shirley 2001) (Figure 1).

The three major types of anthocyanins are pelargonidin (brick red), cyanidin (red), and delphinidin (blue), which are derived from the three dihydroflavonol precursors, dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. These dihydroflavonols are very similar in structure, differing only in the number of hydroxyl groups on the B ring, which is not the site of the DFR enzymatic reaction. Therefore, it is not surprising that DFRs from many species can utilize all three substrates (Forkmann & Heller 1999). However, even though the occurrence of cyanidin and delphinidin is widespread, the elusive substrate specificity of some DFRs plays a key role in controlling pelargonidin biosynthesis (Zufall & Rauscher 2004).

In the present work, we describe the metabolic engineering of *E. coli* for the synthesis of anthocyanins from phenylpropanoic acids and glucose. Prior to the recombinant strain construction, several plant

![Figure 1](image_url). DFR catalyzes the branch point reaction of anthocyanin, proanthocyanin, and phlobaphene biosynthesis.
DFRs were characterized in order to identify DFRs that can robustly synthesize pelargonidin. Furthermore, the biochemical assays were also used to elucidate the efficiency of DFRs to perform flavanone reductase (FNR) activities for the synthesis of the rare flavan-4-ols.

Materials and methods

Chemicals

Cold p-coumaric acid (COU) and caffeic acid (CAF) were purchased from MP Biomedicals Inc Tanaka et al. (1995). Naringenin (NAR) was purchased from Sigma-Aldrich. 7-Hydroxyflavanone, hesperetin, and 5,7-dimethoxyflavanone were purchased from Indofine. 7-Hydroxyflavanone, hesperidin, and 5,7-dimethoxyflavanone were purchased from Alfa Aesar. Flavonoids not commercially available and [14C]-labeled chemicals were synthesized as described by Menting et al. (1994) and Schoenbohm et al. (2000). M9 minimal media consisted of 1X M9 salts, 0.4% (w/v) glucose, 6 mM thiamine, 0.1 μM biotin, 1 μM MgSO4, and 0.1 μM CaCl2. Luria-Bertani (LB) was purchased from Sigma-Aldrich. Eriodictyol (ERI) and DHQ were purchased from Indofine. 7-Hydroxyflavanone, hesperidin, and 5,7-dimethoxyflavanone were purchased from Alfa Aesar. Flavonoids not commercially available and [14C]-labeled chemicals were synthesized as described by Menting et al. (1994) and Schoenbohm et al. (2000). M9 minimal media consisted of 1X M9 salts, 0.4% (w/v) glucose, 6 mM thiamine, 0.1 μM biotin, 1 μM MgSO4, and 0.1 μM CaCl2. Luria-Bertani (LB) was purchased from Sigma. All media were supplemented with appropriate antibiotics according to the selection markers of the plasmids that were contained in the *E. coli* strains.

Plant materials and cDNA clones

Plant tissues used for RNA extraction included the spathe from *Anthurium andraeanum*, flower petals from *Rosa hybrida* cv. “minirose” and *Lilium hybridum* cv. “Star Gazer”, and fruits from *Fragaria ananassa*. DFR cDNA of *Ipomoea nil* was a kind gift from Dr. Shigeru Iiida, National Institute for Basic Biology, Japan. *Arabidopsis thaliana* EST clone SQ147f08-3′ containing the DFR cDNA was a kind gift from Dr. Takakazu Kaneko, Kazusa DNA Research Institute, Japan.

DNA manipulations and heterologous expression

RNAs were isolated from plant tissues using RNeasy Plant Mini Kit (Qiagen). Structural genes were obtained through reverse transcription and polymerase chain reaction (PCR) of RNA fragments. *A. thaliana* dfr was obtained directly through PCR using the EST clone as a template. The accession numbers used for PCR primer design (Table I) were: AY232494 for *A. andraeanum*, D85102 for *R. hybrida* (Mikanagi et al. 2000; Tanaka et al. 1995), AB058641 for *L. hybridum*, AF029685 for *F. ananassa*, AB033294 for *A. thaliana*, and AB006792 for *I. nil*. The DFR protein sequence of *L. hybridum* cultivar “Star Gazer” differed from that of cultivar “Acapulco” and “Montreux” by 6 and 31 amino acids, respectively. DFR cDNA sequence of *Lilium hybridum* “Star Gazer” was deposited to GenBank with the Accession No. AY374471. DFR cDNAs were cloned into pTrcHis2-TOPO (Invitrogen) to create pTrc-DFR plasmid. *E. coli* TOP10F’ strain (Invitrogen) was used for heterologous expression of the DFR proteins.

To allow anthocyanidin synthesis from *E. coli*, five plant genes were introduced into *E. coli* BL21Star (Novagen). FHT cDNA from *Petroselinum crispum* (GenBank Accession No. AY230248) was cloned between the *Pst*I and *Hind*III sites of pRSFDuet-1 vector (Novagen), creating plasmid pRSF-FHT. Subsequently, all DFR cDNAs were cloned individually into pRSF-FHT in between *Kpn*I and *Pac*I to generate pRSF-FHT-DFR. *P. hybrida* cDNAs encoding CHS (GenBank Accession No. AF233638) and CHI (GenBank Accession No. X14589) were cloned into vector pETDuet-1 between *Eco*RV and *Kpn*I, and *Bam*HI and * Psi*I, respectively to yield plasmid pET-CHS-CHI. Parsley 4CL cDNA (GenBank Accession No. X13325) was inserted into plasmid pCDFDuet-1 between *Eco*RV and *Kpn*I, creating pCDF-4CL. The expression cassette contains an individual T7 promoter and a ribosome binding site sequence in front of each gene. Plasmid pRSF-FHT-DFR, pET-CHS-CHI, pCDF-4CL were inserted into BL21Star to create E-color strains.

Recombinant protein expression and enzyme assays

Individual recombinant colonies were grown overnight in LB. The seed culture was used the following day to start a 50-mL LB main culture with an initial

---

**Table I. Primers used for the amplification of dfr cDNA.**

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Forward sequence (5′–3′)</th>
<th>Reverse sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. andraeanum</em></td>
<td>ATGATGCACAGGGGACACCCTGGTG</td>
<td>TCAATGGGCGTGTGTTGCCCGGTG</td>
</tr>
<tr>
<td><em>A. thaliana</em></td>
<td>ATGGTTATGCTGAAAGAGACCCTTG</td>
<td>CTAAGCCACATCGTGGTGCTGAGC</td>
</tr>
<tr>
<td><em>I. nil</em></td>
<td>ATGGTGGATGGGAAAAGGACCCGC</td>
<td>TCAAGGCTTTAAGGAGGCTACC</td>
</tr>
<tr>
<td><em>L. hybrid</em></td>
<td>ATGGGAGATGGGAAAAGGACCCGC</td>
<td>TZZCTGGAAGGACAGGAGACTTG</td>
</tr>
<tr>
<td><em>R. hybrida</em></td>
<td>ATGGCAGCCGGATCGAGGGCACCTTG</td>
<td>TZZGACCTGGACTTTTGACAGCAGC</td>
</tr>
<tr>
<td><em>F. ananassa</em></td>
<td>ATGGGCTTGGGGAGGAGAATCC</td>
<td>CTAACCAGCCTGCGGTTCAG</td>
</tr>
</tbody>
</table>

*Boldface indicates start codon.
*Italics indicate stop codon.*
absorbance at 600 nm ($A_{600}$) of 0.05. This was cultivated at 37°C until $A_{600}$ reached 0.8, at which point the inducer IPTG (isopropyl β-thiogalactoside) at a final concentration of 1 mM was added and the culture incubated with vigorous shaking at 30°C. For DFR in vitro assay, E. coli strains harboring pTrc-DFR were collected after 4 h and frozen at −70°C until use.

In vitro enzyme assays and product identification are described by Martens et al. (2002). Briefly, for protein preparation, E. coli cells were harvested by centrifugation, resuspended in 5 mL of buffer (0.1 M K$_2$HPO$_4$/KH$_2$PO$_4$, pH 6.5), and lysed by sonication. Protein content was determined using the Lowry procedure with crystalline BSA as a standard (Sandermann & Strominger 1972). In vitro DFR and FNR enzymatic reactions were performed using 186 Bq of radiolabeled substrate, and incubation was performed at 30°C for 30 min to 1 h for DFR tests, and 30 min to 2.5 h for FNR tests. Reaction products were separated, identified, and quantified by TLC.

**Biotransformation**

The reduction of four unusual flavanones consisting of flavanone, 7-hydroxyflavanone, hesperetin, and 5,7-dimethoxyflavanone was tested using DFR from A. andraeanum. The biotransformation assay was chosen for that purpose because radio-isotopomer substrates are not available. For this experiment, 4 h after IPTG induction, the DFR expressing E. coli grown in LB were collected by centrifugation and resuspended into a fresh 50-mL M9 culture containing 1 mM IPTG and 10 mg L$^{-1}$ flavanone. Incubation proceeded at 30°C for 24 h, followed by extraction of the culture broth.

For anthocyanin production, E. coli strains grown in LB were collected and resuspended into a fresh 50-mL M9 culture containing 1 mM IPTG and 0.05 mM phenylpropanoic acids. Incubation proceeded at 30°C for 24 h, followed by extraction of the culture broth with an equal volume of ethyl acetate twice. After drying the organic phase in vacuo, spontaneous formation of anthocyanidins from leucoanthocyanidins was performed according to Porter et al. (1986).

**Product quantification and analysis**

Radiolabelled flavonoids were analyzed on precoated TLC cellulose plates (Merck). Leucoanthocyanins and flavan-4-ols were separated from dihydroflavonols and flavanones by chromatography in a chloroform:acetic acid:water (10:9:1) system. The detection and quantification of radioactivity was performed using a Fuji BAS 1000 Bio-Imaging Analyzer. In vitro reaction products were identified using previously published $R_f$ values (Punyasiri et al. 2004), and co-chromatography with reaction products obtained from Gerbera DFR (Martens et al. 2002).

Flavonoids produced from biotransformation with recombinant E. coli were isolated by ethyl acetate extraction of culture media. The organic phase was separated and dried in vacuo. The residues containing the flavonoid products were dissolved in water and analyzed by HPLC using an Agilent HPLC 1100 series instrument with a diode array detector. A reverse phase ZORBAX SB-C18 column (diameter 4.6 × 150 mm) using an acetonitrile (solvent A) and water (solvent B) gradient at a flow rate of 1 mL min$^{-1}$ was used. The HPLC program conditions used were as follows: 10–40% A (0–10 min), 40–60% A (10–15 min), 60% A (15–20 min). Absorbance at 280 nm was monitored in all cases. Retention times of the unnatural flavan-4-ols were compared to 2,4-cis-flavan-4-ol and 2,4-cis-7-hydroxyflavan-4-ol standards prepared as previously described (Pouget et al. 2000). The UV maximum for the various unnatural flavan-4-ols synthesized was: 276 nm for 2,4-cis-flavan-4-ol; 275 nm for 2,4-trans-flavan-4-ol; 281 nm for 2,4-cis-7-hydroxyflavan-4-ol; and 279 nm for 2,4-trans-7-hydroxyflavan-4-ol. Quantification of pelargonidin products was based on the absorbance at 520 nm.

**Results**

**Biochemical properties of recombinant DFR enzymes**

A. andraeanum DFR was used in in vitro assays to determine the optimum pH and temperature of reactions. Monitoring the reduction of radiolabelled substrates in the presence of NADPH, it was determined that the maximum DFR activities towards dihydroflavonols and flavanones were achieved at pH 6–7 (Figure 2A). Unlike previous reports for DFR enzymes heterologously expressed in yeast (Fischer et al. 2003), it was determined that the optimal temperature conditions for all substrates was within the range of 25–30°C (Figure 2B).

For substrate specificity measurement, in vitro reactions of all other recombinant DFRs were performed using the optimal conditions (pH 6.5, 30°C). For each substrate tested, all reactions containing the recombinant DFRs resulted in a single product, identified by the $R_f$ values as described. No products were formed when using crude extract from E. coli harboring empty vector. DHQ and DHM were accepted as substrates by all six recombinant enzymes with similar efficiency.
ranging from 1.0–1.4 pkatal mg⁻¹ protein. However, only the recombinant DFR enzymes derived from *A. andraeanum*, *A. thaliana*, *I. nil*, *R. hybrida*, and *F. ananassa* were able to catalyze the formation of leucopelargonidin (LPg). Recombinant DFRs from *A. andraeanum* and *F. ananassa* utilized DHK very efficiently, with specific activity 21 and 14 pkatal mg⁻¹ protein, respectively. Recombinant DFRs from *I. nil* and *R. hybrida* showed significantly lower activity with DHK, indicated by the lower specific activities of 0.3–0.4 pkatal mg⁻¹ protein. Moreover, the ability of *A. thaliana* DFR to catalyze DHK reduction was extremely low, only measuring to 0.02 pkatal mg⁻¹ protein. No LPg could be detected in assays using *L. hybrid* DFR even when the reaction period was extended and the amount of protein increased up to 100 µg. The specific activities of the recombinant enzymes in reducing dihydroflavonols are summarized in Table II.

From all the DFR enzymatic assays, it was obvious that dihydroflavonol substrates with two or three B-ring hydroxyl groups were reduced with similar efficiency. However, the ability to reduce DHK, the dihydroflavonol substrate with one B-ring hydroxyl group was unique to each DFR enzyme. To obtain correlations of protein primary structure with biochemical data, DFR protein sequence alignments were constructed using Clustal W. Analysis of the central protein domain thought to be responsible for controlling substrate specificity (Johnson et al. 2001) indicated that the two DFR recombinant enzymes with the highest specific activities towards DHK, namely that of *A. andraeanum* and *F. ananassa*, had the amino acid serine and alanine, respectively, at position 133 (Figure 3). In contrast, DFRs with no or low DHK activity have asparagine or aspartic acid at this position (Figure 3). This observation also correlates with the amino acid sequences of *Cymbidium* and *Petunia* DFRs, which have been demonstrated to be unable to metabolize DHK (Figure 3) (Johnson et al. 2001).

**Flavanone reductase activities of recombinant DFR enzymes**

The six recombinant DFR enzymes were also tested in vitro for the reduction of NAR and ERI. The results of the biochemical assays revealed that all of the functionally expressed recombinant DFR enzymes were capable of reducing flavanones. ERI served as a universal substrate for all DFR enzymes, as luteoferol (LTf), the product of ERI reduction, was detected in all assays. Measurement of protein specific activities indicated that ERI was reduced with similar efficiency by all recombinant DFRs, ranging from 0.6 to 0.8 pkatal mg⁻¹ protein. However, it is interesting to note that when NAR was used as the substrate, apiferol (APf) was only

<table>
<thead>
<tr>
<th>Recombinant DFR</th>
<th>DHK</th>
<th>DHQ</th>
<th>DHM</th>
<th>NAR</th>
<th>ERI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. andraeanum</em></td>
<td>21</td>
<td>1.4</td>
<td>1.4</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td><em>A. thaliana</em></td>
<td>0.002</td>
<td>1.3</td>
<td>1.0</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td><em>I. nil</em></td>
<td>0.3</td>
<td>1.3</td>
<td>1.5</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td><em>L. hybrid</em></td>
<td>–</td>
<td>1.3</td>
<td>1.2</td>
<td>–</td>
<td>0.6</td>
</tr>
<tr>
<td><em>R. hybrida</em></td>
<td>0.4</td>
<td>1.0</td>
<td>1.3</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td><em>F. ananassa</em></td>
<td>14</td>
<td>1.2</td>
<td>1.4</td>
<td>0.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>
detected in reactions with recombinant DFR enzymes that were also able to efficiently reduce DHK, namely from *A. andraeanum*, *I. nil*, *R. hybrid*, and *F. ananassa*. Moreover, the synthesis of APf could not be detected in assays using *A. thaliana* DFR. Overall, these assays indicated that flavanones were not as efficiently accepted as dihydroflavonols by DFR enzymes. The biochemical assays demonstrated that all recombinant DFRs tested exhibited FNR activities (specific activities are presented in Table II).

The flexibility of DFR for the reduction of unusual flavanone substrates has not been studied. To investigate substrate flexibility, *A. andraeanum* DFR was tested for the reduction of four unusual flavanones. Biotransformation assays were chosen for that purpose, since these flavanones cannot be synthesized as radio-isotopomers. HPLC results indicated that the recombinant *E. coli* expressing *A. andraeanum* DFR was able to reduce unsubstituted flavanone as well as 7-hydroxyflavanone to the corresponding flavan-4-ols, but not hesperetin or 5,7-dimethoxyflavanone (Figure 4B,E). The first peaks to elute were compared to flavan-4-ol standards (Figure 4A,D) with 2,4-cis stereocenters, and were determined to be 2,4-cis-flavan-4-ol and 2,4-cis-7-hydroxyflavan-4-ol, respectively. The second peaks to elute, the major enzymatic products,
shared nearly identical UV profiles to the 2,4-cis flavan-3-ols, but they were presumed to be the 2,4-trans diastereomers. No product was detected in the biotransformation of *E. coli* harboring an empty vector (Figure 4C,F). To our knowledge, this is the first report that explores the flexibility of a plant DFR in reducing unusual flavanone substrates.

**Production of pelargonidin from *E. coli***

In plants, DFR plays a key role in determining intensity and pigment coloration because its specificity and activities dictate the type and amount of the colorless leucoanthocyanidins. Leucaanthocyanidins undergo subsequent reactions to form the colorful anthocyanidins following a pH shift due to transport to the vacuoles (Nakajima et al. 2001). Anthocyanidins are then enzymatically glycosylated or conjugated with other flavonoid molecules to improve stability (Grotewold 2006). In order to demonstrate the effects of DFRs substrate specificity in controlling pigment formation, a recombinant plant biosynthetic pathway consisting of 4CL, CHS, CHI, FHT, and the various DFRs was introduced in *E. coli*. To achieve optimum transcription and protein synthesis, the T7 phage promoter individually regulated the expression of the plant genes. The multi-gene constructs were then introduced into *E. coli* BL21Star to create various E-color strains.

E-color strains were cultivated in M9 media, and fed with *p*-coumaric acids. After incubation for 24 h, ethyl acetate was used to extract the flavonoids from the culture media. In order to convert the leucaanthocyanidins into anthocyanidins, and to mimic the sequential pH shift in vitro, the dry culture extracts containing leucaanthocyanidins were acidified. Figure 5 shows the different level of pelargonidin generated by the E-color strains when *p*-coumaric acid was incorporated in the fermentations. Table III summarizes the absorbance at 520 nm of pelargonidin synthesized from E-color strains. The highest pelargonidin concentration was derived from the E-color culture harboring the *A. andraeanum* DFR and used as a reference to normalize the absorbance of pelargonidin synthesized from other strains. Overall, the concentrations of pelargonidin synthesized from E-color strains correlated with the specific activity measurement of DFR against DHK (Table II).

**Table III. The relative 520 nm absorbance of pelargonidin synthesized from E-color strains.**

<table>
<thead>
<tr>
<th>DFR variant in E-color</th>
<th>Relative absorbance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. andraeanum</em></td>
<td>100</td>
</tr>
<tr>
<td><em>A. thaliana</em></td>
<td>10</td>
</tr>
<tr>
<td><em>I. nil</em></td>
<td>50</td>
</tr>
<tr>
<td><em>L. hybrid</em></td>
<td>0</td>
</tr>
<tr>
<td><em>R. hybrida</em></td>
<td>50</td>
</tr>
<tr>
<td><em>F. ananassa</em></td>
<td>70</td>
</tr>
</tbody>
</table>

The absorbance of pelargonidin synthesized from E-color harboring *A. andraeanum* was used as the reference value.

**Discussion**

DFR plays a key role in the formation of colored pigments (anthocyanins) and plant-defense molecules (proanthocyanidins). In the present study, we found that all plant DFRs catalyzed the reduction of DHQ and DHM with comparable efficiency. However, the reduction of DHK was selective. The two recombinant DFR enzymes with the highest specific activities towards DHK were derived from *A. andraeanum* and *F. ananassa*, which have a serine and alanine, respectively, at position 133, instead of the consensus amino acid asparagine (Figure 3). It is likely that steric hindrances due to the 133rd amino acid side chain are involved in determining the efficiency of DHK reduction. The chemical structure of leucine is similar to that of asparagines except that it does not carry a bulky side chain. When the asparagine residue at position 134 in *Gerbera DFR* was replaced with leucine (this residue aligned with the 133rd residue in the *A. andraeanum* DFR sequence), a mutant enzyme that only accepted DHK was created (Johnson et al. 2001). Together, these results support the notion that steric hindrance of residue 133 results in inefficient reduction of DHK. We also demonstrated that DHK was accepted by recombinant DFRs isolated from plants that do not accumulate pelargonidin derivatives. For example, pelargonidin is not present in *A. thaliana* plants (Dong et al. 2001), although low DHK reduction activity could be detected using recombinant *A. thaliana* DFR. This result is in agreement with a recent investigation of *A. thaliana* DFR which concluded that the enzyme is capable of utilizing DHK in *A. thaliana* plants only when enzyme in a competing pathway is inactive (Dong et al. 2001). Altogether, these results further implied the presence of metabolic channeling in *A. thaliana*, as...
previously suggested (Burbulis & Winkel-Shirley 1999).

All recombinant proteins that we tested also exhibited FNR activities even though flavanones were not as efficiently reduced as dihydroflavonols. Unlike earlier report on the FNR activity of the DFR enzymes from M. domestica and P. communis (Fischer et al. 2003), all the DFR proteins that could reduce DHK were also able to reduce NAR (Table II). It is, therefore, possible that the catalytic sites for the DFR and FNR activities are the same. No API was detected in assays using A. thaliana DFR, which is not surprising considering its low activity in reducing the natural mono-hydroxylated dihydroflavonol substrate. The flexibility of reducing unusual flavanones was studied using A. andraeanum DFR, since this exhibited the highest activities against the common dihydroflavonol substrates. No activity towards methoxy-substituted flavanones, such as hesperetin and 5,7-dimethoxyflavanone, was detected probably due to steric hindrance by the bulky methoxy groups. The current results support the suggestion that flavonoid methylation occurs further down the flavonoid biosynthetic pathways, rather than during the initial steps (Schröder et al. 2004). Recombinant A. andraeanum DFR was able to reduce unsubstituted (flavanone) or a partially hydroxylated flavanone (7-hydroxyflavanone) to the corresponding tuted (flavanone) or a partially hydroxylated flavanone analog (flavan-4-ol). The biosynthesis of 2,4-trans flavan-4-ols as the major products is consistent with DFR’s exclusive production of (2R,3S,4S)-leucoanthocyanidins that have an analogous C4 stereocenter (Fischer et al. 1988). We speculate that the presence of a neighboring C3 hydroxyl group on the C ring, as with DHK, DHQ and DHM, is responsible for directing the production of only one leucoanthocyanidin stereoisomer. Therefore, the absence of the C3 hydroxyl group, as with flavanones, results in the production of two flavan-4-ol diastereomers. Construction of a DFR crystal structure will further shed light in elucidating the specific residues involved in substrate binding and selectivity.

This work demonstrates that DFR is the controlling step in the conversion of precursor phenylpropanoids to leucoanthocyanidins in recombinant E. coli. The biochemical investigation of various recombinant DFR enzymes has allowed a comprehensive elucidation of DFR substrate specificity. It is likely that each DFR species may require unique conditions to achieve optimum activity. Nevertheless, identification and selection of a DFR enzyme with high-level activity for DHK allowed the robust production of pelargonidin from metabolically engineered E. coli containing a plant pathway. Specifically, we demonstrated that the most robust DFR clones from A. andraeanum or F. ananassa could be incorporated in a pathway engineering strategy to produce anthocyanidins. In light of the current finding, protein engineering of DFR for further improving the recombinant production of a variety of anthocyanins as well as the bioactive flavan-3-ols, and flavan-4-ols will be an important next step. The overproduction of these high-value plant chemicals and their novel derivatives will allow further chemical characterization and rapid testing for pharmaceutical applications.

Acknowledgement

This work was supported by a research grant from the US National Science Foundation (BES-0331404) to MAG Koffas.

References


