Heterologous expression of dihydroflavonol 4-reductases from various plants

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Abstract Dihydroflavonol 4-reductases (DFR) catalyze the stereospecific reduction of dihydroflavonols to the respective flavan 3,4-diols (leucoanthocyanidins) and might also be involved in the reduction of flavanones to flavan-4-ols, which are important intermediates in the 3-deoxyflavonoid pathway. Several cDNA clones encoding DFR have been isolated from different plant species. Despite the important function of these enzymes in the flavonoid pathway, attempts at heterologous expression of cDNA clones in *Escherichia coli* have failed so far. Here, three well known heterologous expression systems for plant-derived genes were tested to obtain the functional protein of DFR from *Gerbera* hybrids. Successful synthesis of an active DFR enzyme was achieved in eukaryotic cells, using either baker's yeast (*Saccharomyces cerevisiae*) or tobacco protoplasts (*Nicotiana tabacum*), transformed with expression vectors containing the open reading frame of *Gerbera* DFR. These expression systems provide useful and powerful tools for rapid biochemical characterization, in particular the substrate specificity, of the increasing number of cloned DFR sequences. Furthermore, this tool allows the stereospecific synthesis of ^{14}C-labeled leucoanthocyanidins in high quality and quantity, which is a prerequisite for detailed biochemical investigation of the less understood enzymatic reactions located downstream of DFR in anthocyanin, catechin and proanthocyanidin biosynthesis.

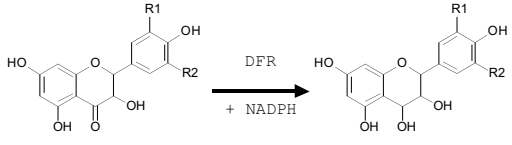
Key words: Flavonoid biosynthesis; Dihydroflavonol 4-reductase; Enzymology; Heterologous expression; Yeast; Tobacco protoplast

1. Introduction

Dihydroflavonol 4-reductase (DFR; EC 1.1.1.219) is a pivotal enzyme of the flavonoid biosynthesis and belongs to the short chain dehydrogenase/reductase or DFR superfamily. Plant-derived members of this superfamily are represented by cinnamoyl CoA:NADP oxidoreductase (CCR; EC 1.2.1.44), cinnamyl alcohol dehydrogenase (EC 1.1.195) and CCR-like proteins with still unknown function. They are all characterized by a similar exon/intron pattern and contain an amino acid sequence motif that seems to be responsible for NADPH binding. Being involved in lignin and flavonoid biosynthesis, these enzymes use precursors supplied by the common phenylpropanoid pathway [1,2].

With NADPH as a cofactor, DFR catalyzes the stereospecific reduction of (2*R*,3*R*)-dihydroflavonols (DHF) to the respective leucoanthocyanidins (2*R*,3*S*,4*S*-flavan-3,4-*cis*-diols) (Fig. 1). The colorless, unstable leucoanthocyanidins are the immediate precursors for the synthesis of anthocyanins, the major water-soluble pigments in flowers and fruits, but they are also precursors for catechins and proanthocyanidins, which are involved in plant resistance and influence food and feed quality of plant products [3].

DFR activity was first demonstrated in protein extracts from *Pseudotsuga menziesii* cell suspension cultures, where the enzyme is related to catechin and proanthocyanidin synthesis [4]. The important role of DFR in anthocyanin formation was later shown with enzyme preparations from flowers of several plant species [5]. It is noteworthy that DFR enzymes exhibit striking substrate specificities, resulting in accumulation of distinct patterns of anthocyanins, catechins and proanthocyanidins. Especially in DFR enzyme preparations of genera of Solanaceae the production of pelargonidin (Pg) derivatives is prevented due to the fact that dihydrokaempferol (DHK) is not accepted as substrate for the formation of leucopelargonidin (LPg) by the protein [5–7]. Therefore, DFR belongs to the key enzymes of the flavonoid pathway, and opens possibilities for metabolic engineering of the pathway [8]. DFR may also exhibit flavanone 4-reductase (FNR) activity catalyzing reduction of flavanones to flavan-4-ols, the key reaction in 3-deoxyflavonoid biosynthesis [9]. The assumption that a single enzyme is involved in the DFR and



R1 = H; R2 = H	dihydrokaempferol (DHK)	leucopelargonidin (LPg)
R1 = OH; R2 = H	dihydroquercetin (DHQ)	leucocyanidin (LCy)
R1 = OH; R2 = OH	dihydromyricetin (DHM)	leucodelphinidin (LDp)

Fig. 1. Enzymatic conversion of dihydroflavonols to leucoanthocyanidins by DFR. The reduction of the skeleton occurs at position C4.

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Abbreviations: DFR, dihydroflavonol 4-reductase; NAR, naringenin; ERI, eriodictyol; DHK, dihydrokaempferol; DHQ, dihydroquercetin; DHM, dihydromyricetin; LPg, leucopelargonidin; LCy, leucocyanidin; LDp, leucodelphinidin

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FNR reactions is further supported by the observation that purified DFR from young buds of *Dahlia variabilis* catalyzed the reduction of both substrates [10], but final proof is still missing. In recent years, the 3-deoxyflavonoid pathway has received considerable attention particularly due to the resistance properties of 3-deoxyflavonoids against fungal and bacterial plant pathogens [11,12] and the antioxidant capacity of these compounds [13].

Since the first cloning of the DFR gene from *Zea mays* by gene tagging with transposable elements [14], DFR sequences have been isolated from many plant species [8]. However, apart from a single report on DFR activity of protein synthesized from the maize cDNA clone using an in vitro transcription/translation system [15], successful functional expression of DFR sequences in heterologous systems has failed so far. Here we report the successful expression of a *Gerbera* DFR cDNA (accession number Z17221) in two different eukaryotic systems leading to synthesis of an active DFR enzyme protein and the application of the yeast system to investigate substrate specificity of recombinant DFR protein from *Gerbera* and five other plant species belonging to different families.

2. Materials and methods

2.1. General

Gerbera hybrida variety 'Terra Regina' was obtained from Terra Nigra (De Kwakel, The Netherlands) and cultivated together with *Dianthus caryophyllus* 'Tanga', *Rosa hybrida* 'Kardinal', *Matthiola incana* 'line 10', *Lycopersicon esculentum* 'line 19' and *Callistephus chinensis* 'line 01' under standard conditions in the greenhouse. Petals of suitable flowering stages and young leaves were harvested, immediately shock frozen in liquid nitrogen and stored at −70°C until further use.

Buffer A: 50 mmol/l Tris–HCl, pH 7.4, 1 mmol/l EDTA, 0.1 mol/l KCl; buffer B: 0.1 mol/l Tris–HCl, pH 7.5; buffer C: 0.1 mol/l Tris–HCl, pH 7.5, containing 28 mmol/l 2-mercaptoethanol. Media: SGI (1 g/l bacto casamino acids, 6.7 g/l yeast nitrogen base, 0.02 g/l tryptophan, 20 g/l glucose); YPGE: 10 g/l yeast extract, 10 g/l peptone, 5 g/l glucose, 3% ethanol. The flavonoid standards naringenin (NAR), eriodictyol (ERI), DHK, dihydroquercetin (DHQ) and dihydromyricitin (DHM) were from our laboratory collection. ^{14}C-Labeled DHK was prepared from its precursor [^{14}C]NAR as described in [16] using recombinant and purified flavanone 3β-hydroxylase enzyme provided by Dr. Richard Lukacin and Frank Wellmann (Marburg, Germany). Using recombinant flavonoid 3′-hydroxylase [^{14}C]NAR and [^{14}C]-DHK were further converted to [^{14}C]ERI and [^{14}C]DHQ, respectively, according to [17]. [^{14}C]DHM was prepared from [^{14}C]DHK using microsomal preparations of *Petunia* as described [18]. All substrates were purified using thin layer chromatography (TLC) methods [19]. Proteins were determined according to [20] with bovine serum albumin as a standard.

For heterologous gene expression, the *Escherichia coli* strain *GI724* and the *Saccharomyces cerevisiae* strain *INV Sc1*, with expression vectors pLEX and pYES2, respectively, were used (Invitrogen, Groningen, The Netherlands). Competent cells of *E. coli* were prepared according to the manufacturer's instructions. Yeast competent cells were prepared with an improved lithium acetate method, according to [21].

2.2. Enzyme preparation from flowers of Gerbera and Matthiola

Preparation of crude extracts and enzyme assays were performed as described in [22]. Enzyme assays of *Matthiola* served as control for DFR reaction. The respective leucoanthocyanidin formed allowed the unequivocal identification of the reaction products obtained from various assays with recombinant proteins by co-chromatography [22].

2.3. PCR cloning and plasmid construction

Total RNA from petals of *G. hybrida* 'Terra Regina', *M. incana* 'line 10', *D. caryophyllus* 'Tanga', *C. chinensis* 'line 01' and young leaves of *L. esculentum* '*line 19*' was isolated according to [23] using 200 mg frozen plant tissue. Poly(A)-tailed RNA of leaves from *Rosa* were obtained using the μMACS mRNA isolation kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instruction. The full-length open reading frames of the respective DFRs were amplified by PCR in a gradient cycler (Hybaid, Heidelberg, Germany), cloned into TOPO T/A cloning vector pCR2.1 (Invitrogen) and confirmed by sequencing in both directions (Medigenomix, Martinsried, Germany). For PCR primers see Table 1. Forward primer contained a *Bam*HI or *Sac*I restriction site immediately followed by the ATG start codon and up to 14 bases of the reading frame. The downstream primer was specific for the 3′ untranslated region of the gene. To facilitate the subcloning of the DFR into the bacterial and yeast expression vectors, the internal *Eco*RI or *Xba*I site downstream of the plasmid pCR2.1 together with the sites introduced by PCR were used. The PCR amplification was performed with Expand High Fidelity enzyme mix according to the manufacturer's instruction (Roche, Mannheim, Germany) and as described in [24]. Plasmids were constructed using standard methods [25].

2.4. Transformation of E. coli and S. cerevisiae with expression plasmids

For expression, the *Gerbera* DFR cDNA fragment was subcloned into the *Bam*HI–*Eco*RI site of pLEX and pYES2 expression vectors, respectively, to afford pLgdfr1 and pYgdfr1. All other fragments were cloned only into pYES2 using suitable restriction sites to give pYmdfr1 (*Matthiola*), pYddfr1 (*Dianthus*), pYrdfr1 (*Rosa*), pYcdfr1 (*Callistephus*) and pYtdfr1 (*Lycopersicon*). The empty expression vector and the expression plasmids containing different DFR genes were used for transformation of competent cells of *E. coli* strain *GI724* (only for pYgdfr1) and *S. cerevisiae* strain *INV Sc1* for subsequent overexpression.

2.5. Isolation and electroporation of tobacco protoplasts

For isolation of protoplasts, leaves of greenhouse-grown *Nicotiana tabacum* 'SR1' were harvested and surface sterilized. Protoplast preparation and subsequent electroporation followed the protocol of [26], except for modifications described below. Instead of counting the cells, their packed cell volume was estimated by weighing at the final stage of their purification. Protoplasts were washed with electroporation buffer 1 [27] and resuspended into the same buffer at a density of 250 mg/ml (about 5 million cells/ml). 0.2 ml of cell suspension was transferred into plastic 1 ml spectrophotometer cuvettes and gently mixed with 10 μl of plasmid DNA in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Plasmid DNA was a mixture of pASH1 (15 μg) and pHTT308 (1 μg), or pHTT308 (1 μg) alone in control electroporations. After electroporation, the cells were allowed to express the plasmids for 28 h in dim light at 23°C, after which they were collected, pelleted and frozen at −70°C. After re-thawing and centrifugation at 10 000 × *g* for 5 min at 4°C the obtained clear supernatant was used as enzyme source.

pASH1 contains the *Gerbera* DFR cDNA under the control of cauliflower mosaic virus 35S transcript promoter (see Section 3). pHTT308 [28] harbors the firefly luciferase cDNA under an enhanced 35S promoter and was used to monitor the success of electroporation. During harvesting of the cells after expression, one tenth of each sample was subjected to assay for luciferase according to [29].

2.6. Heterologous expression in E. coli, S. cerevisiae and tobacco protoplasts

Growth, induction and cell lysis of *E. coli* strain *GI724* transformed with pLEX vector or pLgdfr1 expression plasmid were done as described in the Invitrogen instruction manual 'P_L expression system'. The supernatant fraction after cell lysis was used for enzyme assay.

Growth, induction and cell breakage of *S. cerevisiae* strain *INV Sc1* carrying the pYES2 vector or the pYgdfr1, pYmdfr1, pYddfr1, pYrdfr1, pYcdfr1 and pYtdfr1 plasmid were done as described in [30]. After 16 h incubation at 28°C the cells were harvested by centrifugation, washed with 27 ml buffer A, resuspended in 2.5 ml of buffer B and disrupted with glass beads as described in [30]. After addition of 5 ml buffer B and centrifugation at 12 000 × *g* for 10 min, soluble protein extracts were obtained in the clear supernatant.

2.7. Enzyme assays and product identification

The reaction mixture (final volume 100 μl) containing 0.2 nmol radiolabeled substrate (83 Bq), 10 μl 20 mmol/l NADPH, 10–50 μl protein extract and 40–80 μl buffer C was incubated at 30°C for 25

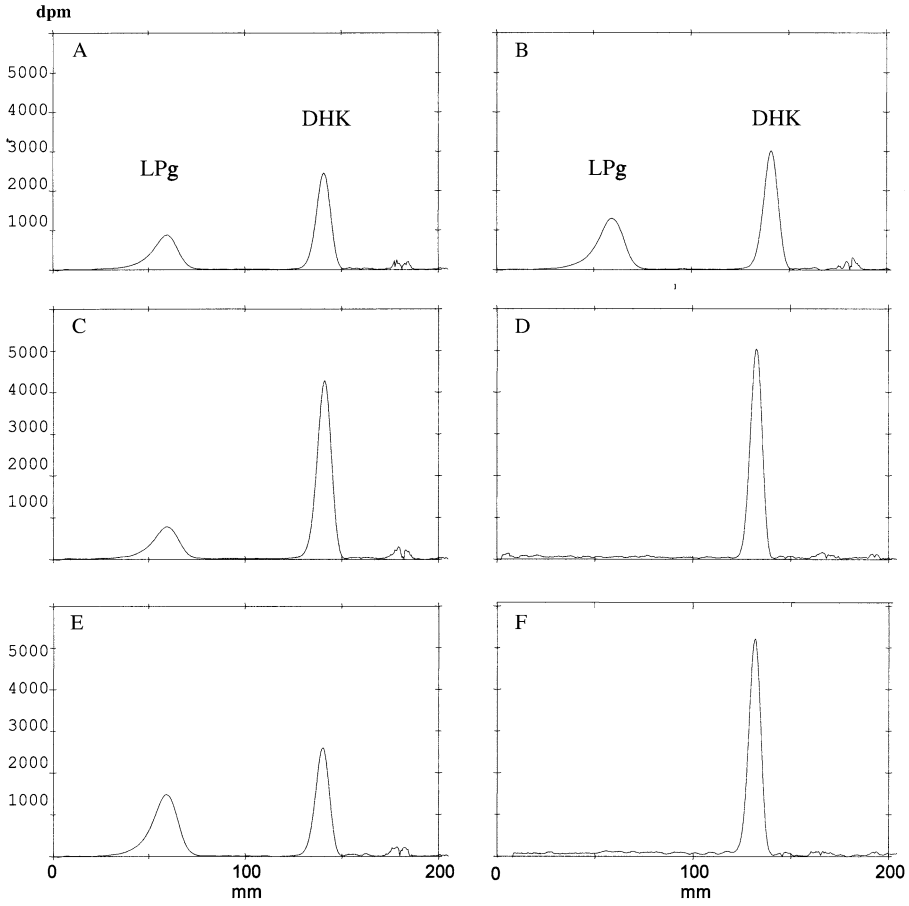

Fig. 2. Detection of substrate (DHK) and product (LPg) after TLC separation by autoradiography. The diverse crude DFR extracts were incubated with [^{14}C]DHK in the presence of NADPH. A: *M. incana* 'line 10' (control). B: Crude extract *Gerbera* 'Terra Regina'. C: *S. cerevisiae* pYgdfr1. D: *S. cerevisiae* pYES2.1. E: Tobacco protoplasts pHTTASH1. F: Tobacco protoplasts pHTT308. Retention time in mm; activity in dpm.

min. For DFR tests the two DHFs and for FNR tests the two flavanones (see Section 2.1) were added separately to the assay. The reaction mixture was extracted twice by adding ethyl acetate {100+100 μl}. Flavonoid extracts were analyzed by TLC on pre-coated cellulose plates (Merck, Darmstadt, Germany). The radioactivity was localized and determined with the Fuji BAS 1000 Bio-Imaging Analyzer. The identity of leucoanthocyanidins formed by the in vitro reaction was confirmed by co-chromatography of the reaction products in three different solvent systems with [^{14}C]leucoanthocyanidins, which was obtained from enzyme assays with *M. incana* 'line 10' [22]. Moreover, by two-dimensional chromatography the formation of the *cis*-form of leucoanthocyanidins was proved [22].

3. Results

3.1. Molecular cloning of Gerbera DFR, subcloning into expression vectors and protein expression

For expression in tobacco protoplasts, the *Gerbera* DFR cDNA was subcloned as a *Bam*HI fragment from pHTT372 [24] into the smaller plasmid vector pHTT300 with a 35S transcript promoter (precursor of pKAH21 [31]) to yield pASH1. For the *E. coli* and yeast expression systems, an end-to-end PCR was performed to create a full-length version

Table 1
PCR primer used for reamplification of the open reading frames of various DFR sequences

Plant species (accession number)	Forward primer	Reverse primer
Gerbera (Z17221)	GerbDFR2H 5′-caaaggatcccAACATGGAAGAGGATTCTCC-3′	GerbDFR2R 5′-TTAGCACATTCCACTCCTTTCTATTGG-3′
Matthiola (not published)	MattDFR1H 5′-caaagagctccATAATGGTTGCTCGCAGAGA-3′	MattDFR1R 5′-CAGTTAATCCAGCTCAGGTTTTG-3′
Callistephus (Z67981)	CallDFR1H 5′-caaaggatcccAACATGAAAGAGGATTCTCC-3′	CallDFR1R 5′-ATCAATTAACTAATTTCACCCCTTCATCG-3′
Dianthus (Z67983)	DianDFR1H 5′-caaagagctccAAGATGGTTTCTAGTACAAT-3′	DianDFR1R 5′-CGAAAACGTGCATCGTTACATCAGG-3′
Lycopersicon (Z18277)	LycDFR1H 5′-caaagagctccAAAATGGCAAGTGAAGCTCA-3′	LycDFR1R 5′-GATCGAGAATATCCATATTTATTGACC-3′
Rosa (D85102)	RosDFR1H 5′-caaaggatcccGCAATGGCATCGGAATCCG-3′	RosDFR1R 5′-CAAACAGAAGAGATACAAATGTTACC-3′

Small letters indicate the restriction site (*Bam*HI or *Sac*I) introduced in the sequence by PCR.

Table 2
Products formed by recombinant DFR proteins from different dihydroflavonols

Recombinant DFR in the assay isolated from [1]flowers, [2]leaves	Anthocyanin pigments found in tissue used[a]	Substrate[a] and conversion to		
		DHK	DHQ	DHM
G. hybrida[1]	Pg, Cy [42]	LPg	LCy	LDp
M. incana[1]	Pg, Cy, Dp [22]	LPg	LCy	LDp
C. chinensis[1]	Pg, Cy, Dp [43]	LPg	LCy	LDp
D. caryophyllus[1]	Pg, Cy [44]	LPg	LCy	LDp
L. esculentum[2]	Cy, Dp [45]	No product formation	LCy	LDp
R. hybrida[2]	Cy, Pn [46]	No product formation	LCy	LDp

[a]Cy: cyanidin; Dp: delphinidin.

of the *Gerbera* DFR open reading frame that completely lacked endogenous 5′ untranslated sequences. PCR primers and cloning vectors were chosen to facilitate further subcloning without additional modification of the amplified fragment. After sequence confirmation the fragment was subcloned into the expression vectors pLEX and pYES2 under the control of the P_L and *gal* promoters, respectively. The obtained expression plasmids pLgdfr1 and pYgdfr1 were used to transform the *E. coli* strain *GI724* and the *S. cerevisiae* strain *INV Sc1*. Empty vectors were used as controls for the expression of recombinant DFR.

3.2. Detection of DFR enzyme activity

DFR activities were measured by using [^{14}C]dihydroflavonols as substrates in the presence of NADPH as a cofactor and the products formed were separated from the substrates by TLC. Incubation of [^{14}C]DHK and NADPH with enzyme preparations from flowers of *Matthiola* 'line 10' and the pink *Gerbera* variety 'Terra Regina' led to the formation of one product with comparable R_f values on TLC (Fig. 2A,B). The identity of the product formed in *Gerbera* enzyme assays was confirmed as LPg as described in Sections 2.2 and 2.7.

When crude enzyme preparations from yeast cells or tobacco protoplasts expressing the *Gerbera* DFR cDNA and NADPH were used for the incubation of [^{14}C]DHK, formation of one product was detected on the TLC radioscan comigrating with the products formed in *Matthiola* and *Gerbera* enzyme assays from flowers (Fig. 2C,E). Product formation increased significantly with protein concentration in the enzyme assay, and no product formation was observed in assays without NADPH. The product formed from DHK was subsequently identified as the *cis*-form of LPg (see Section 2.7). No DFR activity could be detected in enzyme preparations from yeast cells and tobacco protoplasts with empty expression vectors (Fig. 2D,F).

Crude extracts from *E. coli* containing the vector with the complete and properly oriented DFR cDNA sequence showed no DFR activity with either DHK or DHQ as substrate. Even increasing the protein concentration up to 100 μg per assay and/or extending the incubation time up to 1 h did not result in any detectable leucoanthocyanidin formation with *E. coli* extracts.

3.3. Substrate specificity of different recombinant DFR protein

In the past, different plant species served as crude protein sources for biochemical characterization of DFR enzyme activities (for references see [5,6,9]). In order to test the general functionality of the yeast system we overexpressed DFR coding sequences of *M. incana*, *D. caryophyllus*, *L. esculentum*, *C. chinensis* and *R. hybrida* and investigated the substrate specificities of the recombinant DFR protein in vitro.

In addition to *Gerbera* the recombinant protein derived from yeast cells expressing DFR of *Matthiola*, *Callistephus* and *Dianthus* also catalyzes the reduction of [^{14}C]DHK to LPg. In contrast, even by variation of assay conditions including protein amounts, temperature and incubation time, this reaction was not detectable in assays using DFR protein from *Leucopersicon* and *Rosa* (Table 2). But enzyme assays with recombinant DFR protein preparations from all six plant species were able to convert [^{14}C]DHQ and [^{14}C]DHM to leucocyanidin (LCy) and leucodelphinidin (LDp), respectively.

Although 3-deoxyflavonoids have not been described in the six plant species we also tested the reaction with the flavanones NAR and ERI as substrates. Under the assay conditions used here, product formation to the respective 3-deoxyflavonoids, apiforol and luteoforol, could not be observed with any protein extract.

4. Discussion

Nearly all cloned genes involved in the flavonoid pathway have been successfully expressed in different heterologous systems (see [9,32]), including the *Gerbera* sequences encoding chalcone synthase 1 and 3 [33], flavanone 3-hydroxylase (Martens, unpublished), flavone synthase II [34] and the chalcone synthase-like 2-pyrone synthase [35].

Various systems, including bacteria, yeast, insect cells or plant cells, have been used for the in vitro expression of the genes leading to fully active flavonoid enzyme proteins in high yield. This has not only allowed extensive biochemical characterization of the respective enzymes, but also ensured the stereospecific synthesis of flavonoid substrates in profuse amounts.

Although DFR is a key enzyme in the formation of plant pigments and antioxidative, antifungal and antibacterial flavonoid compounds, and enzyme activity has been demonstrated in many plant species in enzymatic studies using crude enzyme preparations, no suitable in vitro expression system has been available so far. Thus, detailed biochemical analyses of the protein, in particular studies on substrate specificity, could be achieved only after time-consuming protein purification procedures [10] or after heterologous expression of DFR clones in transgenic plants [1,36–38].

Our efforts for improvements of heterologous DFR expression led to development of two expression systems, yeast cells or plant protoplasts, where successful in vitro formation of a functional DFR enzyme protein takes place. As in all previous

attempts, efforts to express the DFR cDNA in *E. coli*, the most widely used protein expression system, failed. There may be several possible reasons for failure of *E. coli* systems. Formation of inclusion bodies may prevent accumulation of soluble active protein [39], or different codon usage of *E. coli* may interfere with expression of functional DFR protein. Further, if the DFR enzyme requires posttranslational modification, e.g. by glucosylation or phosphorylation, this would not occur in bacterial cells.

It is important to notice that the recombinant DFR enzymes produced in yeast show the same substrate specificity as the native proteins from crude preparations from flowers and other plant tissues of all six plant species [5]. In agreement with these results and the presence of Pg and cyanidin derivatives in *Gerbera*, *Matthiola*, *Callistephus* and *Dianthus* flowers, DHK and DHQ were found to be substrates for the DFR reaction. The recombinant DFR protein of delphinidin derivative-accumulating species (*Lycopersicon* and *Callistephus*) were able to convert the immediate precursor DHM to LDp. But DHM is also reduced by enzyme extracts from yeast expressing DFR from species naturally lacking delphinidin derivatives (*Gerbera*, *Matthiola*, *Rosa* and *Dianthus*). This might open the possibility for metabolic engineering strategies to obtain blue flowers in *Gerbera*, *Matthiola* and *Rosa* by introducing a suitable flavonoid 3′,5′-hydroxylase in a mutant line accumulating DHK and kaempferol as described for the development of a bluish carnation [8]. As expected, DHK served as substrate in enzyme extracts from the Pg-accumulating species *Gerbera*, *Matthiola*, *Callistephus* and *Dianthus*. However, the protein from expressed *Lycopersicon* and *Rosa* DFR could not reduce this substrate to the respective leucoanthocyanidin, confirming the high substrate specificity with regard to the B-ring substitution pattern and indicating that the products in anthocyanin biosynthesis are strongly dependent on the biochemical properties of this enzyme [5,8].

It has been demonstrated that in *Sinningia cardinalis* and *Columnea* flowers that accumulating 3-deoxyflavonoids a reductase (FNR) similar to DFR catalyzes the NADPH-dependent reduction of flavanones to flavan-4-ols [40,41]. Flavan-4-ols are the direct precursors for the rare 3-deoxyanthocyanins. So far it is an open question whether or not DFR also catalyzes flavanone reduction. However, formation of flavan-4-ols from flavanones was not observed with the recombinant DFR enzymes under the assay conditions used here. This results gave no further hints whether DFR and FNR activities are due to one and the same protein or not. Further biochemical studies using *S. cardinalis* and *Columnea* DFR cDNAs should finally answer this question.

Altogether, DFR expression in the two eukaryotic systems will now allow detailed biochemical studies on reaction mechanism and on substrate specificity of DFR enzymes. This includes the question whether separate DFR and FNR activities are present. Moreover, various DFR cDNAs cloned from further plant species, constructed chimeric DFR sequences or DFRs obtained from site-directed mutagenesis may be investigated [1]. Tobacco protoplasts are especially useful for simultaneous rapid expression of a large number of constructs. The yeast system, on the other hand, is particularly easy to scale up. Expression of active DFR enzyme proteins in high yield will promote the further biochemical and structural characterization of the DFR proteins and facilitate the synthesis of leucoanthocyanidins, the valuable substrates for the further reactions to anthocyanins, catechins and proanthocyanidins, in profuse amounts and independent of the availability of DFR enzyme preparations from plants.

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