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Redirection of anthocyanin synthesis in *Osteospermum hybrida* by a two-enzyme manipulation strategy

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Abstract

Modern biotechnology has developed powerful tools for genetic engineering and flower colours are an excellent object to study possibilities and limitations of engineering strategies. *Osteospermum hybrida* became a popular ornamental plant within the last 20 years. Many cultivars display rose to lilac flower colours mainly based on delphinidin-derived anthocyanins. The predominant synthesis of delphinidin derivatives is referred to a strong endogenous flavonoid 3′,5′-hydroxylase (F3′5′H) activity. Furthermore, since dihydroflavonol 4-reductase (DFR) of *Osteospermum* does not convert dihydrokaempferol (DHK) to leucopelargonidin, synthesis of pelargonidin-based anthocyanins is naturally not realised. In order to redirect anthocyanin biosynthesis in *Osteospermum* towards pelargonidin derivatives, we introduced cDNAs coding for DFRs which efficiently convert DHK to LPg. But neither the expression of *Gerbera hybrida* DFR nor of *Fragaria* × *ananassa* DFR – the latter is characterised by an unusual high substrate preference for DHK – altered anthocyanin composition in flowers of transgenic plants. However, chemical inhibition of F3′5′H activity in ray florets of *dfr* transgenic plants resulted in the accumulation of pelargonidin derivatives. Accordingly, retransformation of a transgenic plant expressing *Gerbera* DFR with a construct for RNAi-mediated suppression of F3′5′H activity resulted in double transgenic plants accumulating predominantly pelargonidin derivatives in flowers.

Keywords: *Osteospermum hybrida*; Asteraceae; Genetic engineering of flower colour; Flavonoid biosynthesis; Anthocyanin; Dihydroflavonol 4-reductase; Flavonoid 3′,5′-hydroxylase; RNAi

1. Introduction

Modern genetic engineering techniques enable to overcome limitations of classical breeding methods. Genes which are not available in the gene pool of a species can be selectively introduced to establish new or modified traits or genes coding for undesired characteristics can be silenced in a targeted manner.

Flower colours are an excellent experimental object for genetic engineering studies since the effects of a gene manipulation strategy can be evaluated visually. Furthermore, broad knowledge about chemistry, biochemistry and molecular biology of the biosynthesis of flavonoids (Fig. 1), the most important group of flower colour pigments, is available as well as the main structural and regulatory genes (Forkmann and Heller, 1999; Winkel-Shirley, 2001; Koes et al., 2005; Grotewold, 2006).

Abbreviations: Cy, cyanidin; Dp, delphinidin; DFR, dihydroflavonol 4-reductase; DHK, dihydrokaempferol; DHQ, dihydroquercetin; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; LCy, leucocyanidin; LPg, leucopelargonidin; LDp, leucodelphinidin; Pg, pelargonidin; RNAi, RNA interference.

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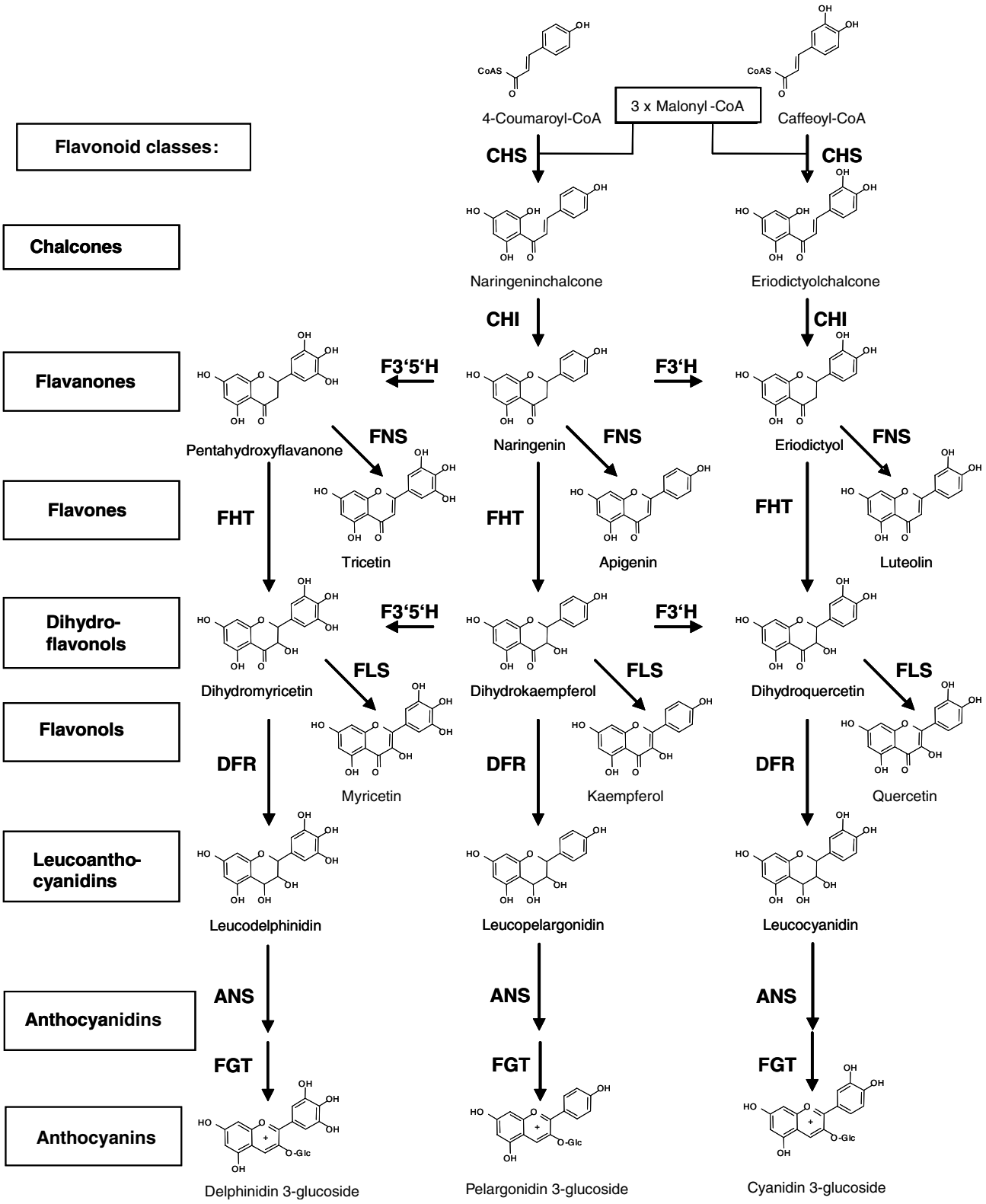

Fig. 1. Schematic presentation of the biosynthesis of important anthocyanins and copigments. Abbreviations for the enzymes are as follows: CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; FHT, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; FGT, flavonoid 3-*O*-glucosyltransferase.

Among flavonoids, the anthocyanins are the main colour-giving class; derivatives of pelargonidin (Pg) provide the basis for orange-red hues, derivatives of cyanidin (Cy) for red hues and derivatives of delphinidin (Dp) for lilac to blue hues. The expression of flower colour is further influenced by the glycosylation and acylation pattern of the

anthocyanidins and by copigmentation effects due to the interaction of anthocyanins with colourless flavonoid classes such as flavones and flavonols (Brouillard, 1983; Grotewold, 2006).

Over-expression or down-regulation of flavonoid genes coding for chalcone synthase (CHS), flavone synthase II (FNS II), flavonol synthase (FLS) or dihydroflavonol 4-reductase (DFR) by sense or antisense transformation approaches led to modified flower colours based on altered amounts of synthesised anthocyanins and/or copigments (reviewed by Tanaka et al., 1998, 2005; Forkmann and Martens, 2001). More recently, RNA interference (RNAi) was applied for the effective suppression of the genes coding for CHS and anthocyanidin synthase (ANS) in *Torenia* (Fukusaki et al., 2004; Nakamura et al., 2006) and for chalcone isomerase (CHI) in tobacco (Nishihara et al., 2005).

The cytochrome P450 proteins flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) play a key role in determining the anthocyanin pattern because F3′H is necessary for the synthesis of Cy-based anthocyanins and F3′5′H for the synthesis of Dp-based anthocyanins (Fig. 1).

F3′5′H cDNAs of for example *Eustoma russellianum* (Shimada et al., 1999), *Petunia hybrida* (Shimada et al., 2001) or *Campanula medium* (Okinaka et al., 2003) have been extensively used for transgenic over-expression. Many efforts have been undertaken in expressing F3′5′H genes in plant species such as rose, carnation or chrysanthemum which naturally lack F3′5′H activity and therefore do not accumulate Dp-derivatives. Most prominent outcome of these trials are purple to violet flower colours due to the induced synthesis of Dp-derivatives in transgenic carnations (Fukui et al., 2003; Tanaka et al., 2005).

In species such as *Petunia*, *Cymbidium* or *Cyclamen*, DFR is not able to efficiently convert dihydrokaempferol (DHK) to leucopelargonidin (LPg), the precursor of Pg-based anthocyanins (Tanaka et al., 2005). Expression of the maize DFR in the DHK-accumulating *Petunia* line "RL01" led to a brick-red flower colour based on Pg-derivatives (Meyer et al., 1987) and represents the starting point for genetic engineering of flower colour.

Cyanic ray florets of *Osteospermum* mainly accumulate derivatives of Dp and, to a lower extent, derivatives of Cy whereas derivatives of Pg are completely lacking (Martens et al., 2003). Enzymatic investigations showed that this anthocyanidin pattern is due to (i) a high activity of F3′5′H leading to the predominant formation of Dp-based anthocyanins and (ii) the inability of the *Osteospermum* DFR to reduce DHK to LPg, the precursor of Pg-based anthocyanins (Martens et al., 2003). Here, we report that the over-expression of cDNAs derived from *Gerbera hybrida* and *Fragaria* × *ananassa* coding for DFR enzymes which are able to efficiently convert DHK to LPg did not alter anthocyanin composition in transgenic *Osteospermum* flowers. But the synthesis of Pg-derivatives could be induced by additional chemical inhibition of F3′5′H activity in flowers of the *dfr* transgenic plants. Accordingly, retransformation of a transgenic plant expressing *Gerbera* DFR with a construct for the RNAi-mediated suppression of F3′5′H activity resulted in double transgenic plants accumulating predominantly Pg-derivatives in flowers.

2. Results

2.1. Expression of Gerbera DFR in Osteospermum

In order to enable formation of Pg-derivatives in *Osteospermum* flowers, we aimed to express a dihydroflavonol 4-reductase (DFR) capable of reducing DHK. For this purpose, the cDNA of *Gerbera hybrida* DFR (GDFR) was inserted into the plant expression vector pBI121. DFR of *Gerbera* has been already shown to efficiently convert dihydrokaempferol (DHK) to leucopelargonidin (LPg) *in vitro* (Martens et al., 2002) and in planta (Helariutta et al., 1993).

The functionality of the resulting construct pBGDFR was tested by introducing the T-DNA into *Petunia hybrida* "RL01" via *Agrobacterium*-mediated transformation. The nearly white flowers of "RL01" accumulate DHK due to lacking activities of flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) and the inability of *Petunia* DFR to convert DHK to LPg (Forkmann and Ruhnau, 1987). As expected from earlier experiments with the maize DFR (Meyer et al., 1987) and GDFR (Helariutta et al., 1993), the flowers of the transgenic "RL01" turned out to be brick-red due to the accumulation of Pg-derivatives (not shown) confirming the functionality of the pBGDFR construct.

Nevertheless, transformation of *Osteospermum hybrida* "Kalanga rosy" with pBGDFR did not result in a visible colour shift in flowers of all 17 obtained transgenic plants (Fig. 2). Furthermore, chemical and spectrophotometrical analysis of ray and disk florets did not show any alteration in the anthocyanin pattern compared to the wild type. Since RT-PCR indicated the successful expression of *gdfr* in transgenic plants, we speculated that DHK is not available as a substrate for GDFR, probably due to a strong endogenous F3′5′H activity. To test this hypothesis, ray florets were treated with tetcyclacis, an inhibitor of F3′H and F3′5′H activity. All 10 treated *gdfr* transgenic plants exhibited a visible colour shift from lilac to orange/red (Fig. 2) and, furthermore, chemical analysis of hydrolysed ray floret extracts clearly demonstrated the presence of Pg (Fig. 3). Tetcyclacis-treated ray florets of untransformed control plants or plants transformed with the pBI121 vector (bearing the *uid*A gene) turned out to be nearly white. Therefore, though GDFR is undoubted present and functional in the transgenic flowers, no DHK is available for the path leading to the formation of Pg-derivatives.

We furthermore prepared crude enzyme extracts and incubated them with DHK and NADPH which are substrate and co-substrate, respectively, for DFR as well as

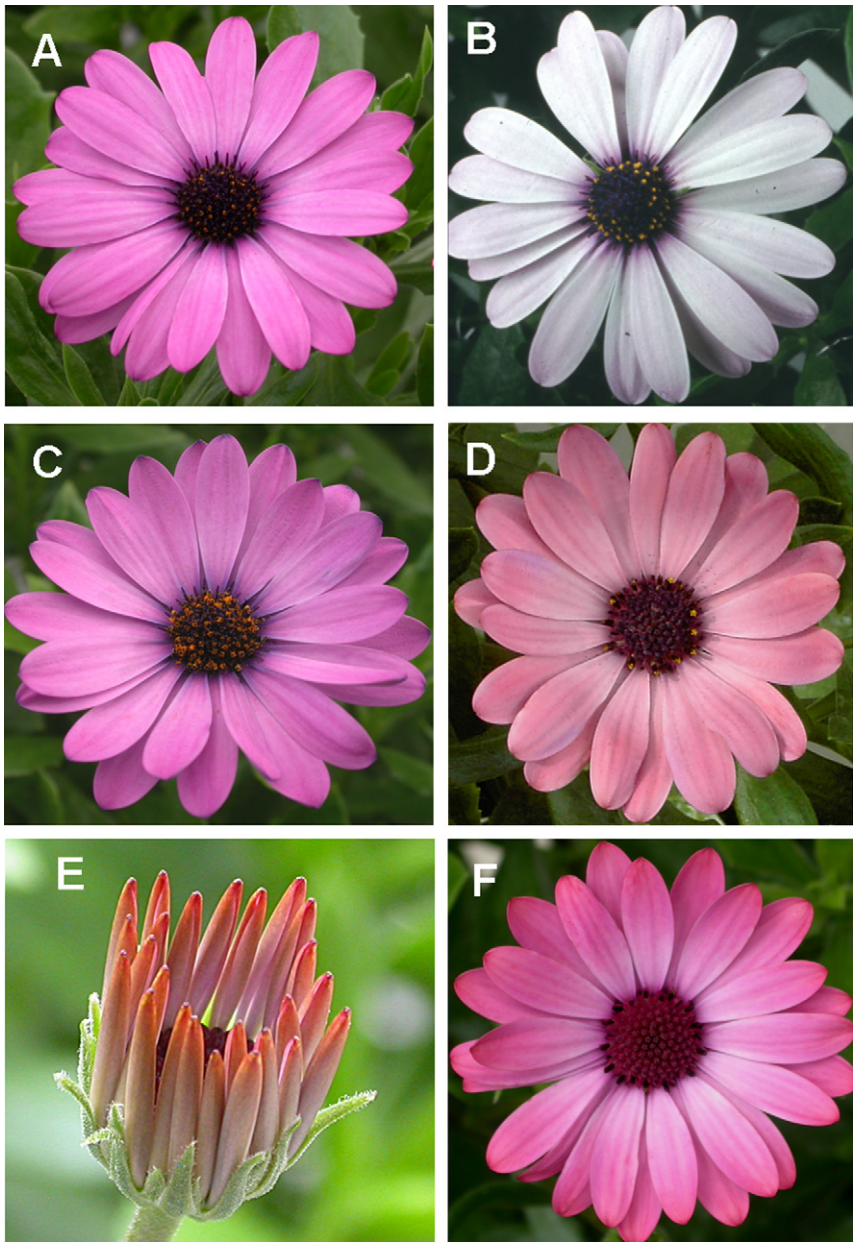

Fig. 2. Flowers of untransformed and transgenic plants. (A) Flower of untransformed plant. (B) Flower of untransformed plant treated with tetcyclacis. (C) *Gdfr*-transgenic flower. (D) *Gdfr*-transgenic flower treated with tetcyclacis. (E) Early flower stage of *gdfr* + RNAi*o*3′5′-transgenic plant. (F) Late flower stage of *gdfr* + RNAi*o*3′5′-transgenic plant.

F3′H and F3′5′H. In a first step, enzyme extracts of *in vitro* plantlets were used because they exhibit only a very weak F3′H activity and no F3′5′H activity preventing competition between DFR and the hydroxylases (see Fig. 1). Hereby, the conversion of DHK to LPg was clearly visible while enzyme extracts of non transgenic controls or plantlets transformed with pBI121 (bearing the *uid*A gene) exhibited only a minor F3′H activity leading to the formation of dihydroquercetin (DHQ). In enzyme assays using a 1:1 ratio of DHK and DHQ as substrates, DHQ was the preferred substrate and the ratio of the synthesised LPg and leucocyanidin (LCy) accounted to around 1:1.5 (Table 1). In agreement with this result, a comparable ratio was obtained with enzyme extracts of *Gerbera* ray florets or recombinant GDFR from heterologous yeast expression.

In contrast to the enzyme extracts of *in vitro* plantlets, incubation of DHK with enzyme extracts derived from ray florets of *gdfr* transgenic plants as well as from non transgenic control plants led to the predominant formation of the 3′- and 3′,5′-hydroxylated products DHQ and dihydromyricetin (DHM) confirming a strong activity of F3′5′H in *Osteospermum* ray florets. The release of 3′,4′-hydroxylated intermediates by F3′5′H is generally observed in *in vitro* enzyme assays with plant protein extracts or recombinant proteins (Kaltenbach et al., 1999; Seitz et al., 2006). But DHQ formation *in vitro* may partly

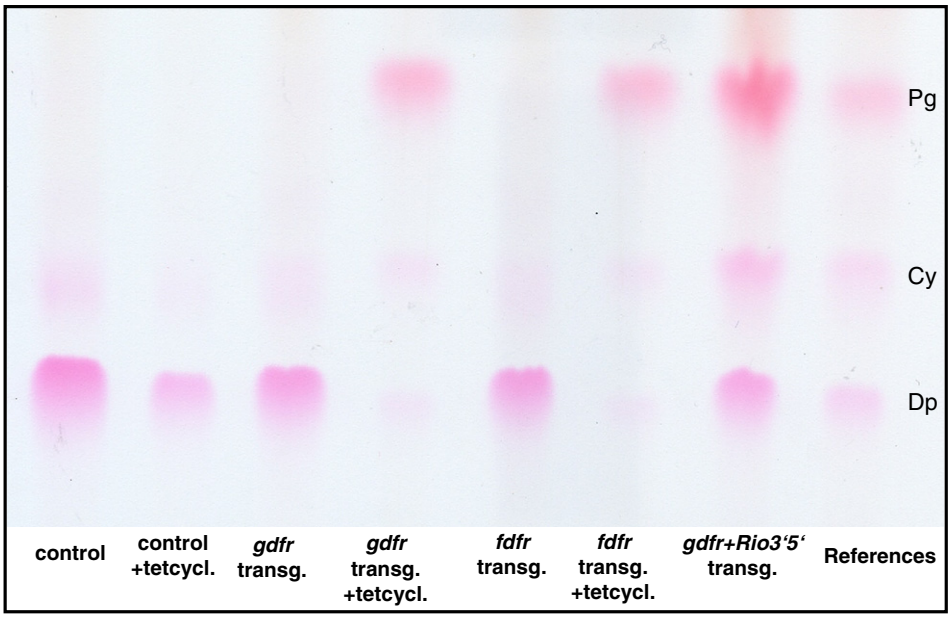

Fig. 3. Anthocyanidins in hydrolysed extracts of ray florets of control and transgenic plants. The hydrolysed extracts were separated by TLC. Abbreviations are as follows: transg., transgenic; +tetcycl., tetcyclacis-treated ray florets; Pg, pelargonidin; Cy, cyanidin; Dp, delphinidin.

be attributed to a weak endogenous F3′H activity since 3′,4′-hydroxylated flavonoids accumulate in low amounts in *Osteospermum* flowers.

The results of the enzymatic investigation allow the conclusion that GDFR is not able to compete with F3′H and F3′5′H for DHK as a substrate in the transgenic plants. Therefore, the lack of Pg-derivatives in *gdfr* transgenic plants can be explained by a strong activity of F3′5′H and/or metabolic channelling preventing the accumulation of DHK as a substrate for the production of LPg by DFR. Alternatively, activity of GDFR with DHK may be too weak to efficiently compete with the B-ring hydroxylases.

Based on these results, we set up two further approaches. The first approach consisted in the use of the DFR of *Fragaria* × *ananassa* which was shown to exhibit an unusual high specifity for DHK as a substrate (C. Gosch, University of Technology, Vienna, Austria, personal com.). As a second approach, we aimed to retransform the *gdfr* transgenic plants with an appropriate RNAi-construct for the suppression of F3′5′H activity.

2.2. Expression of Fragaria DFR in Osteospermum

The cDNA of *Fragaria* × *ananassa* DFR (FDFR) was inserted into pBI121. Ten transgenic plants exhibiting PCR-proven integration and expression of *fdfr* were obtained. But as found for *gdfr* transgenic plants, no colour shift was observed in flowers of all *fdfr* transgenic plants and no Pg-derivatives could be detected in hydrolysed flower extracts (Fig. 3). However, as observed with the *gdfr* transgenic plants, treatment of transgenic ray florets with tetcyclacis resulted in a clear colour change from lilac to orange/red due to the accumulation of Pg-derivatives.

Again, DFR activity with DHK as a substrate could be clearly detected in enzyme extracts of *in vitro* plantlets. In enzyme assays using a 1:1-ratio of DHK and DHQ as substrates, DHK turned out to be the clearly preferred substrate resulting in a 4:1-ratio of LPg and LCy as products (Table 1). The ratio obtained for the recombinant FDFR from yeast expression was lower (3:1). Such discrepancies between enzyme extracts of plant tissues and yeast cultures have been observed with other DFRs, too, and are probably caused by the yeast expression system (Seitz et al., unpublished). However, in either case the observed product ratio stresses the unusual high substrate specifity for DHK of FDFR compared to GDFR or other DFRs which prefer DHQ (Forkmann and Heller, 1999). But despite this preference of FDFR for DHK, enzyme extracts of ray florets of *fdfr* transgenic plants again converted DHK exclusively to DHQ and DHM and not to LPg, indicating that a strong activity of F3′5′H and/or metabolic channelling prevents accumulation of DHK as a substrate for FDFR.

2.3. RNAi-mediated silencing of F3′5′H in GDFR transgenic plants

In order to retransform a *gdfr* transgenic plant, we prepared an RNAi-construct (pRNAiO3′5′) containing a 544 bp fragment of the *Osteospermum* F3′5′H cDNA in sense and antisense orientation. To prevent cross silencing of other cytochrome P450 genes, the chosen fragment is located 3′ off the region coding for the prolin-rich membrane anchor region and 5′ off the region coding for the motifs involved in oxygen and heme binding which exhibit relative high sequence conservation (Schuler and Werck-Reichhart, 2003).

Table 1
DFR activity in enzyme extracts of various sources

Conversion (%) of:	Enzyme source						
	Gerbera ray florets	Recombinant GDFR from yeast expression	Recombinant FDFR from yeast expression	*Osteo.* ray florets	*Osteo. in vitro* plantlets	*Osteo. gdfr* transgenic *in vitro* plantlets	*Osteo. fdfr* transgenic *in vitro* plantlets
DHK to LPg	31.4	30.7	34.6	0	0	23.1	20.1
DHQ to LCy	45.7	49.4	11.4	40.5	0	32.6	4.9
Ratio LPg/LCy	0.69	0.62	3.04	–	–	0.71	4.1

Crude enzyme extracts of plant tissues or yeast cultures were incubated with a 1:1-ratio of DHK and DHQ as a substrate. The enzyme assays were repeated at least two times.

The pFGC1008 vector was chosen for the construct because it bears a marker gene for the selection of transgenic cells by hygromycin and therefore allows the retransformation of already kanamycin-resistant transgenic plants.

A *gdfr* transgenic plant was retransformed and 5 PCR-proven double transgenic plants (*gdfr* + RNAi*o*3′5′ transgenic) have been obtained so far. The flowers of all these plants exhibited a clear colour change (Fig. 2). Both the disk florets and the lower part of the developing ray florets appeared orange whereas the upper part of developed ray florets was more reddish. Accordingly, in hydrolysed extracts, Pg was clearly identified as main anthocyanidin (Fig. 3). However, beside traces of Cy, Dp is clearly present, too.

In contrast to untransformed as well as to *gdfr* and *fdfr* transgenic plants, enzyme extracts prepared from ray florets of the double transgenic plants clearly converted DHK to LPg whereas the conversion of DHK to DHQ and DHM was substantially reduced indicating a successful downregulation of F3′5′H activity. However, the silencing is not complete and the remaining F3′5′H activity can be hold responsible for the observed formation of Dp-derivatives (see below).

For a more detailed characterisation of the course of anthocyanin accumulation, we quantified anthocyanin content in three developmental stages (stage 1: ray florets out of bud, beginning of pigmentation; stage 2: ray florets stretched and pigmented; stage 3: ray florets fully developed) of an untransformed control plant and a *gdfr* + RNAi*o*3′5′ transgenic plant. In flowers of the untransformed plant, the content of Dp-derivatives was markedly higher in stages 2 and 3 compared to stage 1 (Fig. 4). In flowers of the double transgenic plant, the content of Pg-derivatives was uniform throughout the three stages while the content of Dp-derivatives was constantly increasing. The ratio of Pg- to Dp-derived anthocyanins amounted to 1.6 in stage 3 compared to 5.6 and 2.8 in stages 1 and 2, respectively. This decreasing ratio of Pg- to Dp-derivatives is consistent with the colour shift from orange to reddish during flower development

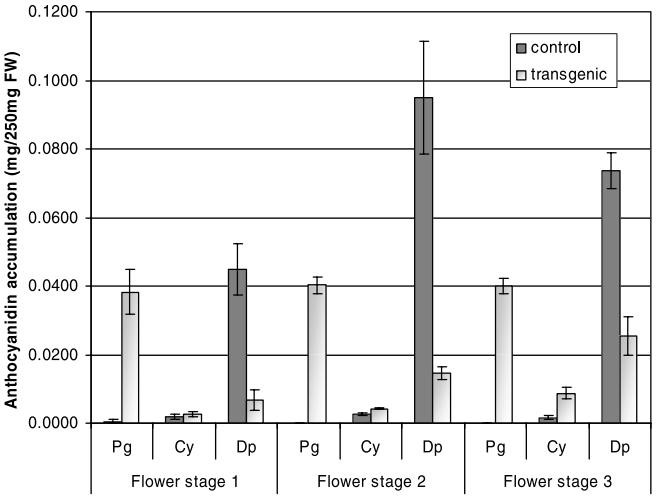

Fig. 4. Amounts of anthocyanidins in flower extracts of three developmental stages. Concentrations were estimated after HPLC separation. Quantities are expressed as cyanidin equivalents. Abbreviations are as follows: Pg, pelargonidin; Cy, cyanidin; Dp, delphinidin.

(see Fig. 2). The content of Cy-derived anthocyanins is higher in the transgenic flowers but still remains negligible.

We furthermore measured F3′5′H activity in the three stages (Fig. 5). The specific activity found for the transgenic plant relative to those found for the control amount to 7.7%, 5.2% and 7.6% in stages 1, 2, and 3, respectively. Therefore, the degree of *f*3′5′*h* silencing is high and constant throughout flower development and does not explain the relatively high contents of Dp-derivatives which are 15.3% in stages 1 and 2 and 34.4% in stage 3 compared to the wild type. This discrepancy can be explained with a more efficient metabolic interaction of the remaining F3′5′H protein with the endogenous flavonoid enzymes compared to the introduced GDFR. In this respect, the around 2-fold increased specific F3′5′H activity in stage 3 (1.42 pKat/mg protein) compared to stages 1 and 2 (around 0.75 pKat/mg protein) is correlated with the 2.2-fold increase in Dp-derivatives in the late flower stage.

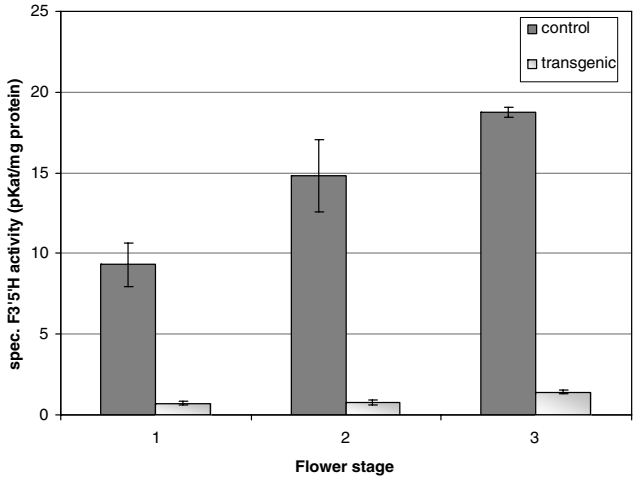

Fig. 5. Measurement of F3′5′H activity in ray floret enzyme extracts of 3 developmental stages. Crude enzyme extracts were incubated with naringenin as a substrate. At least three independent repetitions were performed. The activity was calculated as the sum of the 3′,4′- and 3′,4′,5′-hydroxylated products eriodictyol and pentahydroxyflavanone, respectively, since the release of 3′,4′-hydroxylated products is generally observed in F3′5′H *in vitro* assays. However, a smaller portion of the eriodictyol formation may be attributed to a weak endogenous F3′H activity in *Osteospermum* ray florets.

3. Discussion

We found that neither the expression of Gerbera DFR nor of *Fragaria* DFR led to the synthesis of Pg-derivatives in flowers of transgenic *Osteospermum* plants per se but additionally required chemical inhibition of F3′5′H activity. This clearly demonstrated the necessity of both the presence of suitable DFR activity and the suppression of competing F3′5′H activity for the redirection of anthocyanin biosynthesis. Consequently, the approach comprising the retransformation of a *gdfr* transgenic plant with an RNAi-construct for the down-regulation of *f3′5′h* resulted in double transgenic plants displaying flowers accumulating mainly Pg-derivatives.

DFR activity with DHK as a substrate was clearly proven in enzyme extracts of *gdfr* and *fdfr* transgenic *in vitro* plantlets. Hereby, as found for many other DFRs, enzyme extracts of *gdfr* transgenic plants accepted DHQ as a substrate more efficiently than DHK (Forkmann und Heller, 1999). In contrast, enzyme extracts of *fdfr* transgenic plants had a drastically higher activity with DHK as a substrate compared to DHQ, a substrate preference that has been rarely found so far (Xie et al., 2004).

However, even the expression of *Fragaria* DFR with this unusual high specifity for DHK did not lead to formation of Pg-derivatives in *Osteospermum* flowers. This indicates that 3′, 5′-hydroxylation of DHK is much more efficient than the conversion to LPg by the introduced DFRs. An alternative explanation relies on the key role of F3′H/F3′5′H as membrane-bound enzymes are supposed to play in substrate channelling in macromolecular complexes formed by the flavonoid enzymes (Winkel-Shirley, 1999). In such a complex, the intermediate flux may efficiently be directed from FHT to F3′H/F3′5′H and subsequently to DFR so that a physical contact between DHK and DFR is prevented. Consequently, even if the introduced DFR is able to both interact with the enzyme complex and utilise DHK as a substrate, formation of LPg will not occur. A more efficient metabolic interaction of F3′5′H with the other endogenous flavonoid enzymes compared to the introduced GDFR can explain the unexpected high accumulation of Dp-derivatives in double transgenic plants though F3′5′H activity was drastically reduced. Despite the drastic reduction in the amount of F3′5′H protein and the respective enzyme activity the formation of 3′, 4′,5′-hydroxylated anthocyanins apparently is still advantaged.

In agreement with these considerations, a variety of examples exists where formation of Pg-derivatives does not occur even if the endogenous DFR is able to convert DHK to LPg. For example in red-coloured flowers of chrysanthemum and sunflowers exclusively Cy-derivatives accumulate though DFR of both species is able to convert DHK to LPg (Schwinn et al., 1993; Seitz et al., unpublished). Analogous situations are found not only in flowers and regarding anthocyanin biosynthesis. In *Arabidopsis* seeds, only F3′H mutants accumulate Pg-derivatives, whereas seeds with functional F3′H exclusively accumulate Cy-derivatives and even the over-expression of maize DFR did not lead to Pg-derivatives (Dong et al., 2001). In condensed tannin synthesis, the products of DFR activity, the leucoanthocyanidins, are the precursors of propelargonidin, procyanidin and prodelphinidin units which form the condensed tannin polymers. However, in *Lotus corniculatus*, the expression of the DHK-accepting DFR of *Anthirrhinum majus* did not elevate the level of propelargonidin units in leave and stem tissues (Robbins et al., 2005).

The RNAi strategy employed in this study has led to five individual transformants with a homogenous and markedly *f3′5′h*-silenced phenotype. In contrast, attempts to downregulate *f3′5′h* in *Osteospermum* flowers by a co-suppression strategy resulted only in a small portion of transgenic plants exhibiting a slight decrease in anthocyanin accumulation (Seitz et al., unpublished). Generally, RNAi-based approaches lead to a high frequency of silenced transformants. More than 50% and even up to 100% RNAi-mediated gene-silenced transformants have been observed with *Arabidopsis*, tobacco, cotton, rice or wheat (Smith et al., 2000; Wesley et al., 2001; Travella et al., 2006). In *Torenia*, RNAi-mediated downregulation of the anthocyanidine synthase gene resulted in more than 50% of the transgenic plants exhibiting the expected phenotype (white flowers), compared to 1% and 0% of the plants transformed with an antisense or sense construct, respectively (Nakamura et al., 2006). Similar results were obtained with *Arabidopsis* or potato (Chuang and Meyerowitz, 2000; Andersson et al., 2006).

Though RNAi-induced gene-silencing can be normally efficiently achieved, the degree of gene silencing can vary

between lacking and weak to a complete knockout, a phenomenon mainly attributed to positional effects of the T-DNA insertion events and/or transgene copy number (Travella et al., 2006). With respect to biotechnological flower colour breeding this variability can be used for the selection of various phenotypes (Nakamura et al., 2006). Regarding *Osteospermum*, evaluation of further *gdfr* + RNAi*o*3′5′ plants could lead to the discovery of plants with a stronger or even complete *f*3′5′*h* knockout. The flowers of such plants should exhibit a low or lacking accumulation of Dp-derivatives even at later flower stages and therefore continuously display an orange colour. Alternatively, the CaMV35S promoter of the RNAi construct could be strengthened in order elevate expression levels or replaced by another promoter. The latter may be convenient since we used the 35S promoter for the DFR as well as the RNAi construct which may have led to a partial silencing and prevented higher expression levels.

We found elevated but still minor amounts of Cy-based anthocyanins in the double transgenic plants. In agreement with this observation, co-suppression of *f*3′5′*h* in *Petunia* and *Torenia* drastically decreased the accumulation of Dp-derivatives and increased the level of Cy-derivatives (Shimada et al., 2001; Suzuki et al., 2000). This can be explained with a higher availability of DHK as a substrate for F3′H. However, in all cases the level of Cy-derivatives remained substantially lower than the total anthocyanin level in the untransformed plants. In *Torenia*, the additional expression of a F3′H cDNA in a *f*3′5′*h* silenced plant was necessary to achieve a sufficient colouration based on Cy-derivatives (Ueyama et al., 2002). With respect to *Osteospermum*, accumulation of higher amounts of Cy-derivatives in the *gdfr* + RNAi*o*3′5′ plants may be prevented by the competition between F3′H and the introduced GDFR for DHK as a substrate. Consequently, down-regulation of F3′5′H in *Osteospermum* (without introduced DFR activity for DHK as a substrate) should induce formation of higher amounts of Cy-derivatives. A partial downregulation should lead to a Cy/Dp-mixture type, a complete down-regulation to a pure Cy-type. However, the additional over-expression of a F3′H gene may be required in order to obtain sufficient levels of Cy-derivatives.

4. Experimental

4.1. Binary vector construction and transformation in *A. tumefaciens*

The cDNA of DFR of *Gerbera hybrida* (GDFR; GenBank accession no. Z17221) was kindly provided by Dr. S. Martens (Philipps-University, Marburg, Germany), that of *Fragaria* × *ananassa* (FDFR; GenBank accession no. AF029685) by C. Gosch (University of Technology, Vienna, Austria). The *uid*A gene of the binary vector pBI121 (GenBank accession no. AF485783) under control of the CaMV35S promoter and NOS terminator was replaced by the full length DFR cDNAs using the *Xba*I and *Sac*I and the *Bam*HI and *Sac*I restriction sites, respectively. The inserts with appropriate overhangs were generated by sticky ends PCR (Zheng, 1998). The primers used for cloning are shown in Table 2. The resulting constructs were named pBGDFR and pBFDFR. pBI121 carries the *npt* II gene under control of the NOS promoter allowing the selection of kanamycin resistant transgenic plants.

The RNAi vector pFGC1008 was obtained from ChromDB (http://www.chromdb.org/) via the Arabidopsis Resource Center (http://www.arabidopsis.org/). A 544 bp fragment (position 267–810 of the coding sequence) of the *Osteospermum hybrida* F3′5′H cDNA (GenBank accession no. ABB43031) was inserted in sense and antisense orientation according to the instructions given at the ChromDB website. The sense and antisense fragment are separated by a 322 bp spacer and are under control of the CaMV35S promoter and the OCS terminator. The resulting construct was named pRNAiO3′5′. The pFGC1008 vector bears a hygromycin resistance gene under the control of the MAS promoter for the selection of transgenic plants.

The prepared constructs were transformed into *A. tumefaciens* strain EHA105 by electroporation.

4.2. Plant material and transformation

Osteospermum hybrida cv. ‘‘Kalanga rosy’’ was used for *Agrobacterium*-mediated transformation. Establishment of an *in vitro* culture and propagation system, the infection of leaf discs with *A. tumefaciens* strain EHA105 as well as the

Table 2
Primers used for construct design

Construct name	Primers used	Sequence (5′–3′)
pBGDFR	GDFR-forward	AACATGGAAGAGGATTCTCC
	GDFR-reverse	ATTCCACTCCTTTCTATTGG
pBFDFR	FDFR-forward	GCAGCTCTAACTAAAGCA
	FDFR-reverse	GATAACTTCGACTGGATCGGAG
pRNAiO3′5′	O3′5′-forward	AGGGATAATGACTTGAA
	O3′5′-reverse	GCTCAACATGTCATCAT

Primer sequences are shown without the 5′-overhangs necessary for the creation of sticky ends.

regeneration and rooting of transgenic shoots and the acclimatisation under greenhouse conditions was performed as described in detail by Mercuri et al. (2002) with following modifications: a two days pre-culture period was employed; for a more effective induction of regeneration, leaf discs were cultivated on medium containing 2 μg/ml α-naphtyl acetic acid and 1 μg/ml N^6-benzyladenine during preculture, co-culture and the first two weeks of selection; for kanamycin-based selection of transgenic cells 50 μg/ml of the antibiotic were used.

For the selection of transgenic cells by hygromycin, 3 μg/ml were used during callus formation and 5 μg/ml during shoot formation.

Putative transgenic plants were verified regarding successful integration and expression of the transgenes by standard PCR-techniques.

At least 12 months were needed from the infection of the leaf explants by *A. tumefaciens* to flowering transgenic greenhouse plants. The transgenic plants were cultivated under greenhouse conditions suitable for *Osteospermum* (Hass-Tschirschke, 1996) and maintained by cuttings.

4.3. Flavonoid analysis

Extraction and analysis of flavonoids by spectrophotometrical means and thin layer chromatography (TLC) was performed according to standard procedures (Harborne, 1973; Markham, 1982). Authentic references were used for the identification of flavonoid compounds. The effect of chemical inhibition of F3′H and F3′5′H activity on flavonoid composition and colour of *Osteospermum* flowers was investigated by the application of tetcyclacis (1 mg/ml sterile water), a kind gift of Dr. W. Rademacher (BASF, Ludwigshafen, Germany). For HPLC anaylsis, the anthocyanins were separated on a column (250 × 4 mm i.d.) prepacked with Hypersil ODS, 3-μm particle size, following a stepwise gradient using mixtures of solvent A (formic acid, 5% in water) and solvent B (methanol, gradient grade) from 95:5 (v/v) to 10:90 (v/v) with a flow rate of 0.5 ml/min. The gradient profile (% B in A) used was as follows: 0–5 min, isocratic, 5% B; 5–15 min, 5–10% B; 15–30 min, isocratic, 10% B; 30–50 min, 10–15% B; 50–70 min, isocratic, 15% B; 70–85 min, 15–20% B; 85–95 min, isocratic, 20% B; 95–110 min, 20–25% B; 110–140 min, 25–30% B; 140–160 min, 30–40% B; 160–175 min, 40–50% B; 175–190 min, 50–90% B. The anthocyanins were detected at 540 nm and quantified as cyanidin equivalents. Peaks were classified into derivatives of pelargonidin, cyanidin and delphinidin by means of their maximal absorbance at 500 nm, 520 nm and above 526 nm, respectively, using a diode array detector.

4.4. Enzymatic analysis

The preparation of crude enzyme extracts was performed at 4 °C. 100 mg ray florets were ground with 100 mg seasand, 50 mg dowex 1 × 2 and 1 ml 0.1 M KPi (containing 40 mmol/l Na-ascorbate), pH 7.0. The homogenate was centrifuged (16,000 × rpm, 10 min) and the resulting supernatant used as enzyme source. The protein content was determined by standard procedures (Bradford, 1976). Heterologous yeast expression of the DFR cDNAs of *Gerbera hybrida* and *Fragaria* × *ananassa* and recombinant enzyme preparation was performed as described in Martens et al. (2002).

Crude enzyme extracts (10–40 μl) were incubated with 0.02 nmol [^{14}C]-labelled flavonoid substrates (50 Bq) from our lab collection, 10 μl 20 mmol/l NADPH and 50–80 μl 0.1 M KPi, pH 7.0 in a total volume of 100 μl. DHK, DHQ, or a 1:1 combination of both was used as substrates for DFR assays, NAR or DHK, respectively, for F3′5′H assays. NAR and DHK were found to be equal substrates for *Osteospermum* F3′5′H. Although F3′5′H is a membrane-bound enzyme, crude enzyme extracts of *Osteospermum* flowers exhibit similar hydroxylation activities compared to microsomal preparations. To ensure linearity of the reaction with protein concentration and incubation time, we used not more than 40 μg protein per assay and 20 min of incubation at 30 °C for F3′5′H and DFR assays with plant enzyme extracts and maximal 10 μg protein and 10 min of incubation at 30 °C for DFR assays with recombinant DFRs from yeast expression. Therefore, under the chosen conditions, not more than 50% of the substrate(s) was converted to the respective product(s). In the F3′5′H assays, we observed the formation of both 3′,4′- and 3′,4′,5′-hydroxylated products. The marked release of 3′,4′-hydroxylated intermediates by F3′5′H is generally observed in *in vitro* enzyme assays with plant protein extracts or recombinant proteins and strongly depends on the incubation conditions (Kaltenbach et al., 1999; Seitz et al., 2006). Therefore, the F3′5′H activities were calculated using the sum of 3′,4′- and 3′,4′,5′-hydroxylated products. Under the chosen conditions, a ca. 2:1 ratio of 3′,4′- to 3′,4′,5′-hydroxylated products was observed. Using higher protein amounts, an almost exclusive formation of 3′,4′,5′-hydroxylated products can be achieved. However, formation of 3′,4′-hydroxylated products *in vitro* may partly be attributed to a weak endogenous F3′H activity since 3′,4′-hydroxylated flavonoids accumulate in minor amounts in *Osteospermum* flowers.

After incubation, the enzyme assays were extracted with 500 μl EtOAc. The upper phase was chromatographed on a precoated cellulose plate (Merck, Darmstadt, Germany) in following solvent system: $CHCl_3$:HOAc:H_2O (10:9:1). Radioactivity was localized and determined with the FUJI BAS 1000 Bio-Imaging Analyzer (Tokyo, Japan).

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