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Flavonoid synthesis in *Petunia hybrida*: partial characterization of dihydroflavonol-4-reductase genes

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Abstract

In this paper we describe the organization and expression of the genes encoding the flavonoid-biosynthetic enzyme dihydroflavonol-4-reductase (DFR) in *Petunia hybrida*. A nearly full-size DFR cDNA clone (1.5 kb), isolated from a corolla-specific cDNA library was compared at the nucleotide level with the *pallida* gene from *Antirrhinum majus* and at the amino acid level with enzymes encoded by the *pallida* gene and the A1 gene from *Zea mays*.

The *P. hybrida* and *A. majus* DFR genes transcribed in flowers contain 5 introns, at identical positions; the three introns of the A1 gene from *Z. mays* coincide with the first three introns of the other two species. *P. hybrida* line V30 harbours three DFR genes (A, B, C) which were mapped by RFLP analysis on three different chromosomes (IV, II and VI respectively).

Steady-state levels of DFR mRNA in the line V30 follow the same pattern during development as chalcone synthase (CHS) and chalcone flavanone isomerase (CHI) mRNA. Six mutants that accumulate dihydroflavonols in mature flowers were subjected to Northern blot analysis for the presence of DFR mRNA. Five of these mutants lack detectable levels of DFR mRNA. Four of these five also show drastically reduced levels of activity for the enzyme UDPG: flavonoid-3-*O*-glucosyltransferase (UFGT), which carries out the next step in flavonoid biosynthesis; these mutants might be considered as containing lesions in regulatory genes, controlling the expression of the structural genes in this part of the flavonoid biosynthetic pathway. Only the an6 mutant shows no detectable DFR mRNA but a wild-type level for UFGT activity. Since both an6 and DFR-A are located on chromosome IV and DFR-A is transcribed in floral tissues, it is postulated that the An6 locus contains the DFR structural gene. The an9 mutant shows a wild-type level of DFR mRNA and a wild-type UFGT activity.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X15536 (*pallida*) and X15537 (DFR-A).

Introduction

Flavonoid synthesis in *Petunia hybrida* offers an excellent model system to study the regulation of gene expression in higher plants. Some 35 genes have been described that influence the synthesis of flavonoids either quantitatively or qualitatively (for an overview see [37]). Furthermore, a number of enzyme activities, involved in the pathway have been described along with their genetic control [16, 17, 32]. This allows the system to be analysed in detail at the molecular level [21, 22, 26, 31, 34].

In *P. hybrida* the conversion of dihydroflavonols into anthocyanins is the most complex step in flavonoid biosynthesis. Six genes are known that block the production of anthocyanins and lead to the accumulation of dihydroflavonols in flowers, of the recessive homozygote: an1, an2, an6, an9, an10 and an11 [4, 9, Cornu and Farcy, personal communication]. Plants, homozygous recessive for either an1, an6, an10 or an11 are white-flowered; an2 recessives can show colour in the flower veins and an9 recessives have very pale self-coloured flowers.

Dihydroflavonols are cytosolic, whereas anthocyanins are found in the vacuole exclusively [19, 35]. From this one might presume that at least some of the genes mentioned above might be involved in transport of flavonoid intermediates over the tonoplast. It is not known whether the chemical conversions are interlocked with the transport process itself. However, the methyltransferases that accomplish the last possible modifications of the anthocyanins are known to be cytosolic [20]. Alternatively, some of the genes might be regulatory genes, modifying the activity of the flavonoid enzymes by controlling biosynthetic gene expression.

At least two enzyme activities are needed for the conversion of dihydroflavonols into anthocyanins: dihydroflavonol-4-reductase (DFR) and UDPglucose: flavonoid-3-*O*-glucosyltransferase (UFGT; cf. Fig. 1). The expected intermediates, leucoanthocyanidins (or flavan-3,4-diols, cf. [16]) and anthocyanidins have not been demonstrated *in vivo* in *P. hybrida*; they are both unstable in aqueous solutions. According to Hrazdina [19], flavan-3,4-diol is converted into anthocyanidin via the intermediate flavene-3. UFGT activity has been described in some detail, together with its genetic control [11,12].

DFR enzyme activity has been described for a number of species. In *Matthiola incana* the enzyme preferentially catalyses the conversion of dihydrokaempferol to leucopelargonidin [17]. In *Z. mays* DFR enzyme activity was tested with dihydroquercetin as a substrate which is converted into leucocyanidin [30]. Only pelargonidin and cyanidin derivatives are found in *Z. mays* and *Antirrhinum majus*. In contrast, cyanidin and delphinidin derivatives are the main anthocyanins in *P. hybrida* [9]. The DFR enzyme from *P. hybrida* catalyses the reduction of dihydroquercetin to leucocyanidin and, more efficiently, of dihydromyricetin to leucodelphinidin, whereas reduction of dihydrokaempferol to leucopelargonidin has not been observed [7]. Thus, the nature of the

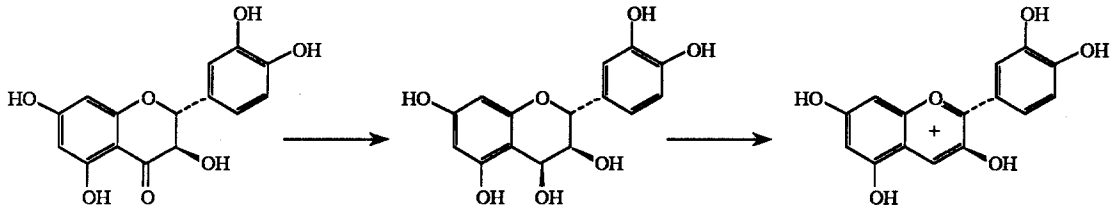

Fig. 1. The conversion of dihydroquercetin into cyanidin. Dihydroflavonol reductase (DFR) reduces dihydroquercetin to leucocyanidin, which is further reduced to cyanidin. Cyanidin is subsequently converted to an 3-*O*-glucoside by UDPG: flavonoid-3-*O*-glucosyltransferase (UFGT; not shown here).

Table 1. Substrate specificity of the DFR enzyme (as tested in an *in vitro* assay) and product accumulation in *Zea mays*, *Antirrhinum majus* and *Petunia hybrida*.

	Substrates tested			Anthocyanins as found in coloured tissues		
	DHK[1]	DHQ	DHM	pel	cya	del
Z. mays	nt	x	nt	x	x	nf
A. majus	nt	nt	nt	x	x	nf
P. hybrida	nt	x	x	nf	x	x

[1] DHK = dihydrokaempferol; DHQ = dihydroquercetin; DHM = dihydromyricetin; pel = pelargonidin; cya = cyanidin; del = delphinidin; nt = not tested; nf = not found; x = activity present/anthocyanin found.

anthocyanins produced is at least partly determined by the substrate specificity of the DFR enzyme (cf. Table 1).

DFR genes have been described in *A.* and *Z. mays*. The phenotypically recognizable mutations have been called *pallida* and *a1* respectively [1, 35]. Alleles of both genes have been cloned [25, 27, 33]. We used part of the *pallida* gene as a probe for the isolation of the *P. hybrida* and *A. majus* DFR cDNA clones and the *P. hybrida* genomic clones. In this paper we describe the molecular organization of the DFR genes and the genetic control of DFR expression.

Materials and methods

Petunia strains

All strains used have been maintained as inbred stocks for at least four generations, more frequently for over ten generations. The phenotypic markers used for RFLP analysis are briefly described in Gerats *et al.* [14]. At least one marker gene was analysed for every linkage group: Hf1 (I) is involved in the conversion of dihydrokaempferol and dihydroquercetin into dihydromyricetin; Fl (II) induces a tenfold increase in flavonol content; Ht1 (III) is involved in the conversion of dihydrokaempferol into dihydroquercetin; as a chromosome IV marker we used a Pac3 probe, kindly provided by M. McLean [23]; Po(V) influences pollen colour; Rt(VI) governs the conversion of anthocyanin-3-glucosides into their rutinoside counterparts; An4 (VII) controls pollen colour.

RFLPs for the DFR-A and -C genes were detected in the lines M5 and W146, using *Eco* RI. The backcross (M5 × W146) × W146 was analysed for the phenotypic markers Ht1 (III), Po (V) and Rt (VI). Mini prep DNA of single progeny plants was digested with *Eco* RI and the resulting filters were hybridized with the λDFR-A1 probe. The (V23 × R51) F1 hybrid was back-crossed to both parental strains [36, 23]; an RFLP for DFR-B was detected using *Xba*II. Besides linkage to the chromosome II standard marker, Fl, we also detected linkage with a chalcone synthase gene located in chromosome II, CHS-G [22].

Isolation of DNA from P. hybrida

A 5-g aliquot of leaves was frozen in liquid nitrogen and homogenized with mortar and pestle. The fine powder obtained was thawed in 15 ml buffer (100 mM Tris, 100 mM NaCl, 20 mM EDTA, 1% (w/v) sarkosyl; pH 8.0) and 1.5 ml of 20% SDS and left at 37 °C for 2 min. The solution was extracted twice with an equal volume of phenol (saturated with 10 mM Tris, 1 mM EDTA, pH 8.0) and once with an equal volume of phenol : chloroform (1 : 1). The aqueous phase was precipitated with 2 volumes of 96% ethanol and left at room temperature for at least 30 min. The precipitate was centrifuged for 20 min at 4000 rpm and the resulting pellet dried briefly in a desiccator. The pellet was resuspended in 10 ml TE (10 mM Tris, 1 mM EDTA; pH 8.0). The DNA was purified on a CsCl gradient.

Miniprep DNA was isolated according to Dellaporta *et al.* [3].

Isolation of total RNA

Total RNA was isolated as described by van Tunen *et al.* [34].

Isolation of plasmid and phage DNA. Plasmid and phage DNA were extracted as described by Koes *et al.* [21].

Preparation of genomic and cDNA libraries. We used the libraries as prepared by Koes *et al.* [21] and by van Tunen *et al.* [34].

Construction of physical maps of λ EMBL-3. Construction of physical maps of λ EMBL-3 inserts were made by analysis of partial restriction-enzyme digest specifically labeled at the cosR site [29].

DNA sequence analysis. The dideoxy chain termination method was used for sequencing M13 clones. A full length cDNA clone of DFR was subcloned into the *Eco* RI site of M13mp18 in both orientations. An overlapping set of deletion clones was generated by exonuclease digestion [18] and used for subsequent sequencing of the cDNA.

Southern blotting and hybridization. For Southern blotting 2–5 μg of restricted DNA was run on a 0.8% agarose gel and blotted on GeneScreen Plus and hybridized according to the protocol of the supplier (NEN, 1983 Instruction Manual). Southern blotting and hybridization of cloned DNA fragments was performed according to the supplier, using Hybond-N membranes (Amersham). Preparation of probes was carried out following standard procedures.

Northern blotting and hybridization. For Northern blotting 10 μg of total RNA was electrophoresed on agarose/formaldehyde gels stained with ethidium bromide [24], blotted on GeneScreen Plus and hybridized according to the protocol of the supplier (NEN, 1983 Instruction Manual).

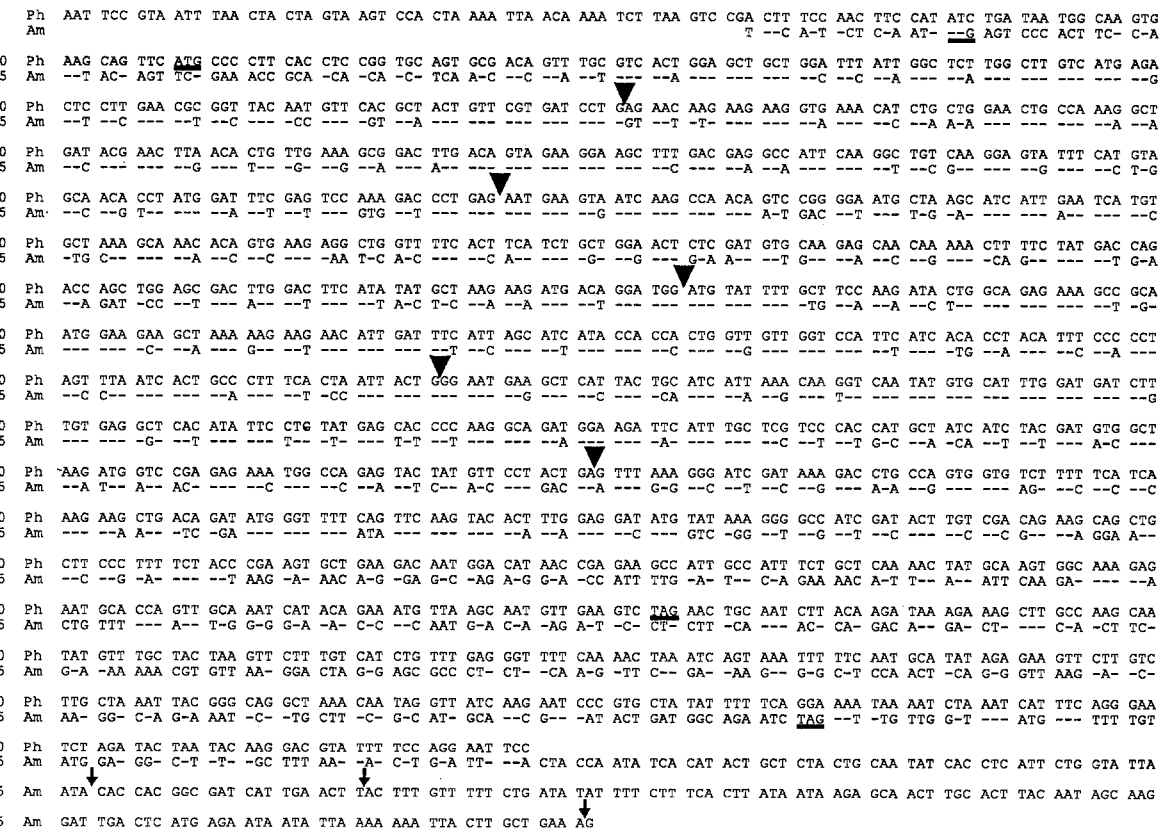

Fig. 2. Nucleotide sequence of λDFR-A1 and comparison with the *Antirrhinum majus* cDNA sequence of the *pallida* gene. Identical bases are indicated by a dash. Intron positions, which are identical for both genes are indicated by arrowheads. The ATG start codon and the stop codons are underlined. Three polyadenylation sites in the *A. majus* sequence are indicated by arrows.

Results

Cloning and sequence analysis of DFR cDNA from A. majus *and* P. hybrida

A cDNA library was prepared in λgt10 from developing flowerbuds (15–25 mm long) of *A. majus* strain JI522. This library was screened with a 1.4 kb *Eco* RI-*Bam* HI fragment from the genomic clone of the *pallida* gene [25], which includes transcribed sequences of the gene. 87 Positive clones were identified from an unamplified library consisting of 100000 pfu. Twelve of the plaques hybridizing most strongly to the probe were purified and restriction maps were derived. The clones were all of different length and appeared to be derived from the same transcript as evidenced by restriction mapping and hybridization behaviour (results not shown). One cDNA clone, which was nearly full-length (1.4 kb), was subcloned into pUC18 and subsequently sequenced. The 3′ ends of three other cDNA clones were also sequenced. Three different polyadenylation sites were identified from this analysis. The cDNA sequence is presented in Fig. 2.

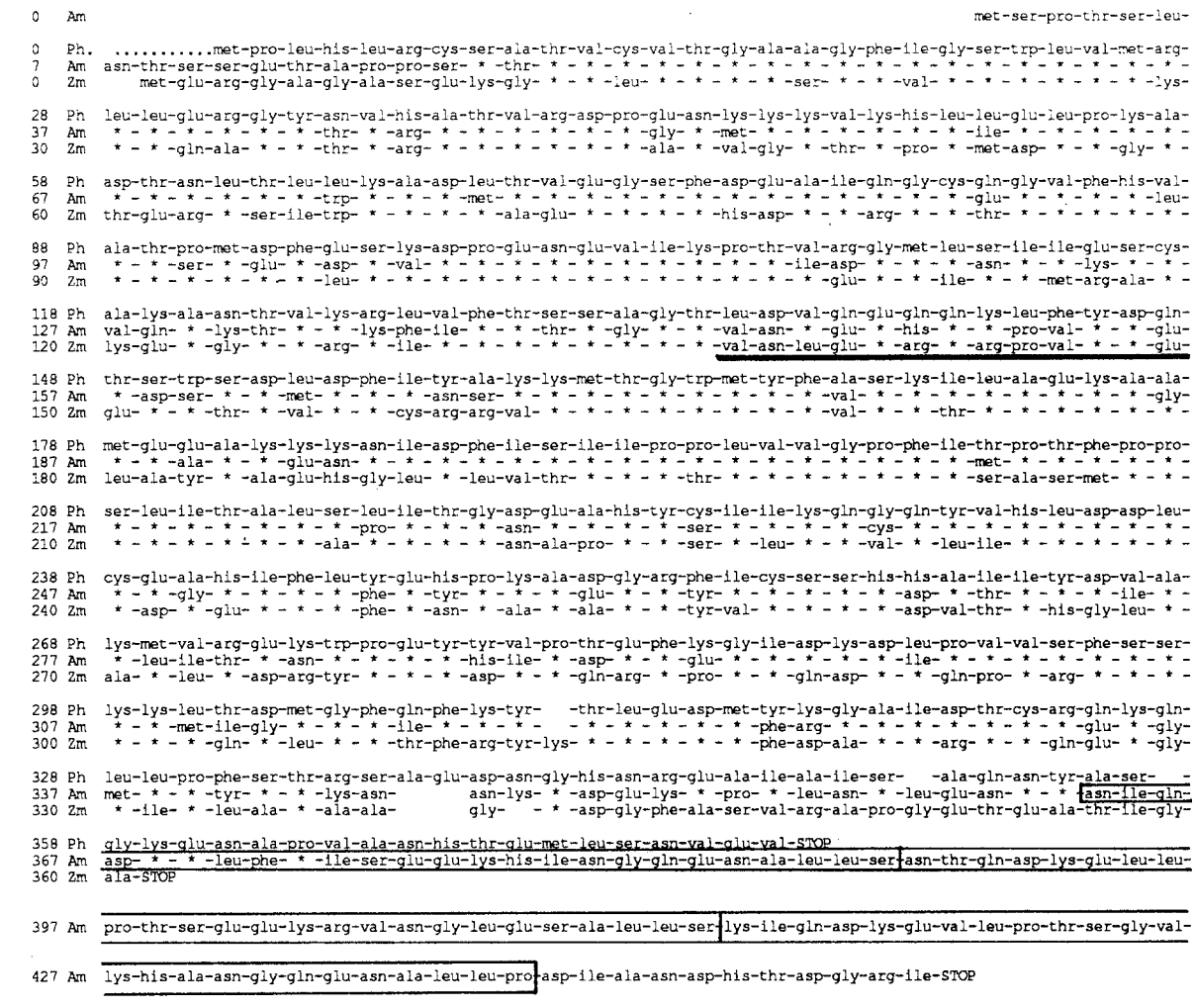

Fig. 3. Comparison of deduced DFR aminoacid sequences for *P. hybrida*, *A. majus* and *Z. mays* [33]. Amino acids identical to those of the *Petunia* sequence are indicated by an asterisk. A stretch of 13 amino acids, which shows high homology in *A. majus* and *Z. mays* as opposed to *P. hybrida* is underlined (see text). A threefold repetition of 25 amino acids at the C-terminal end in the *A. majus* sequence is boxed.

A λgt11 cDNA library, prepared from corolla tissue of flowerbuds of *P. hybrida* line R27 [34] was screened with the same 1.4 kb fragment of the *pallida* gene [25]. Fourteen clones hybridizing to this probe were purified. Thirteen of these contained an insert of nearly the same size (1.5 kb), whereas one clone contained an insert of around 1.6 kb. Sequence analysis of the 5' and 3' ends of both types of clones revealed that the smaller cDNA insert (λDFR-A1) was nearly full-length, whereas the larger cDNA insert had an extended 3' region and was shorter at the 5' end. Sequence analysis showed no differences between the clones; moreover, all fourteen clones show an identical hybridization behaviour, indicating that these clones represent transcripts of the same gene. The λDFR-A1 clone was subcloned into M13mp18. Deletion clones were obtained by exonuclease III treatment [18] and subsequently sequenced. The cDNA sequence is presented in Fig. 2. The translational start and stop codons were deduced from the DNA sequence.

Comparison of the derived amino acid sequence (Fig. 3) for the DFR genes from *P. hybrida*, *A. majus* and *Z. mays* [33] revealed high homology between the three genes, except at the beginning and the end of the protein sequence. The homology between the *Petunia* and *Antirrhinum* sequences was greater than the homology of either to the maize sequence, as might be predicted. The only exception is a stretch of 13 amino acids between the *Petunia* residues 134 and 148, where the *Zea* and *Antirrhinum* sequences are more closely related.

Identification and isolation of P. hybrida *DFR genes*

Flavonoid genes can occur as multigene families in *P. hybrida*. At least eight complete chalcone synthase genes and a number of gene fragments are present in the line V30 [32]. For the chalcone flavanone isomerase two genes were found [34]. To estimate the degree of amplification for the DFR gene, genomic DNA of line V30 was digested with the restriction endonuclease *Eco* RI and subjected to Southern blot analysis, with the λDFR-A1 clone as a probe (Fig. 4). From the

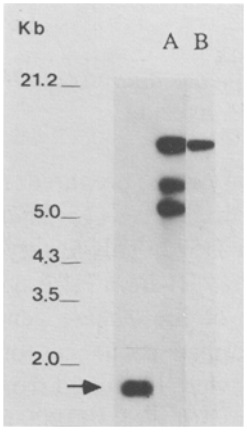

Fig. 4. Southern blot analysis of genomic V30 DNA digested with *Eco* RI. The filter was washed sequentially under low (2× SSC; 1% SDS; 60 °C; lane A) and high (0.1× SSC; 1% SDS; 65 °C; lane B) stringency conditions after blotting and hybridization with the λDFR-A1 probe. A single copy reconstruction is indicated by the arrow.

results of this experiment we estimate the number of DFR genes as three per haploid genome. Only one genomic fragment hybridizes strongly to the cDNA probe as evidenced by washing at different stringencies. To document this further, Sau3A partially digested V30 DNA was cloned into EMBL-3 [34]. The resulting library of approximately 250000 pfu was screened using the λDFR-A1 clone as a probe. Eight positive clones were isolated and purified. These clones were subjected to restriction enzyme digestion, blotted and hybridized to the λDFR-A1 clone. By washing at different stringencies (Fig. 5) and by comparing restriction maps (results not shown) it is concluded that the isolated clones represent three classes of genomic DFR inserts. On the basis of these results and of Southern blot analysis of V30 DNA (Fig. 4), we concluded that the haploid V30 genome contains three DFR genes: A, B and C. All isolated cDNA clones show an identical hybridization behaviour and are most homologous to the DFR-A gene.

Genomic localization of the P. hybrida *DFR genes by RFLP mapping*

The genomic localization of a gene has traditionally been restricted to genes for which pheno-

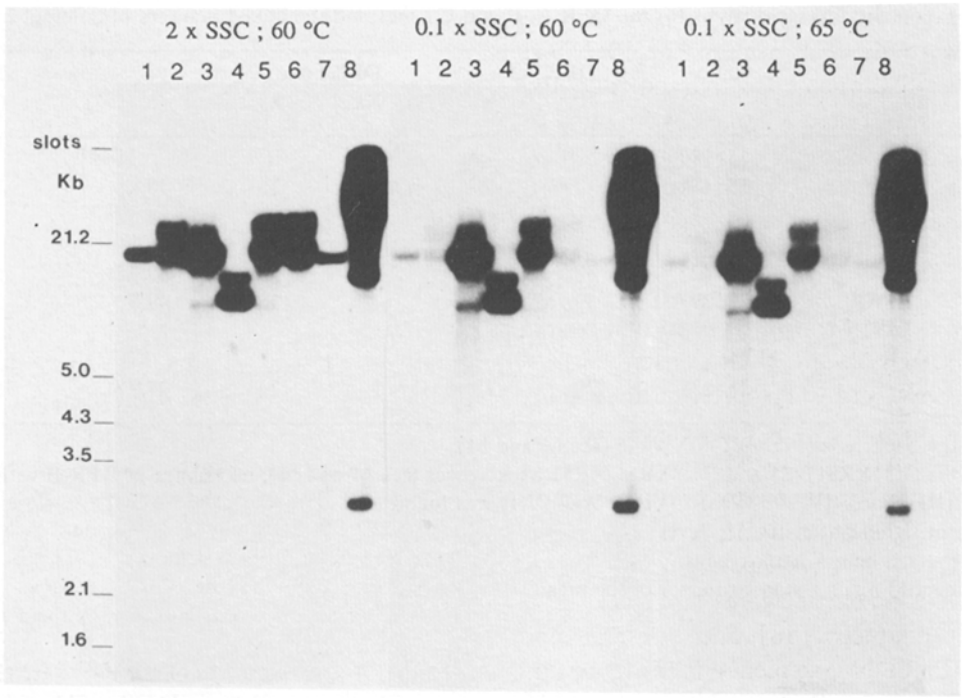

Fig. 5. Southern blot analysis of 8 genomic V30 clones, digested with *Sal* I. The filter was sequentially washed at different stringencies after blotting and hybridization with the λDFR-A1 probe. The isolated clones represent 3 classes: DFR-A (numbers 3, 4 and 8), DFR-B (5) and DFR-C (1, 2, 6 and 7). The λDFR-A1 clone is most homologous to the DFR-A gene.

typically contrasting alleles were available. RFLP (restriction fragment length polymorphism) mapping provides a very powerful technique for linkage analysis that is independent of the expression of the gene to be localized. The system is based on the occurrence of point mutations, deletions, inversions and/or insertions. As a result a cleavage site for a given restriction enzyme can be induced or destroyed or can change place in the genomic DNA. Restriction of two different lines with that enzyme will then show different sized bands after hybridization with a probe that is (partly) homologous to the piece of DNA that contains the cleavage site difference. Once an RFLP is detected in two lines, it can be analysed as any other phenotypic trait.

The genomic localization of the three DFR genes was analysed in backcrosses that segregated for a number of phenotypic markers (see Materials and methods). The relevant RFLP data are presented in Table 2. The gene from which the cDNAs were derived, DFR-A, appeared to be linked with an actin gene, located in chromosome IV [23]. DFR-B was found to be linked with the marker for chromosome II, Fl. The gene least homologous to the cDNA, DFR-C, was located in chromosome VI, linked with the flavonoid gene Rt.

Transcription analysis of DFR genes in V30 and in dihydroflavonol-accumulating mutants

The steady-state levels of DFR mRNA, at different stages of corolla, tube and anther development, in line V30 were measured (Fig. 6). In young flower buds the transcript could not be detected, but levels increase markedly during flower development to a maximum just prior to unfolding of the petals. In contrast, the maximum steady-state level of DFR mRNA in anthers is reached at an early stage of development. The induction pattern of DFR mRNA is similar to that found for the mRNAs of both chalcone

Table 2. Segregation and linkage analysis for the DFR-A, B and C genes and the linked markers of different backcrosses.

Chrom.	Marker	Ratio	DFR het[d]		DFR hom[d]		χ^2_{2x2}	Distance (cM ± SEM)[e]
			Xx	xx	Xx	xx		
	DFR-A[a]	15 : 18[c]						
IV	PAC3[a]	15 : 18[c]	13	2	2	16	18.8	12.1 ± 5.6
	DFR-B[b]	43 : 42[c]						
II	FL[b]	43 : 42[c]	38	5	5	37	49.7	11.8 ± 3.5
II	CHS-G[b]	36 : 39[c]	32	3	4	36	49.6	9.3 ± 3.4
	DFR-C[a]	21 : 27[c]						
VI	Rt[a]	25 : 23[c]	20	1	5	22	27.9	12.5 ± 4.8

[a] Analysed in the backcross (M5XW146)XW146 (see M and M).
[b] Analysed in the (V23XR51)V23 and (V23XR51)XR51 backcrosses (see M and M); no linkage of DFR-B with the markers Hf1 (I), Ht1 (III), Pac3 (IV), Po (V), Rt (VI) or An4 (VII) was found
[c] Segregation not significant at the 5% level.
[d] het = heterozygous; hom = homozygous
[e] cM ± SEM: centiMorgan ± standard error of the mean.

synthase and chalcone-flavanone isomerase [34].

Six mutants are known in *P. hybrida* that accumulate dihydroflavonols. They are white-flowered (an1, an6, an10 and an11), show a veining pattern (an2) or a flush of colour (an9). Limbs of flower buds of the same developmental stage as those containing the highest amounts of DFR mRNA in the line V30, were harvested from all mutant lines and their DFR mRNA levels determined by Northern blot analysis. Surprisingly, in five out of six mutants no DFR mRNA could be detected (Fig. 7). Only the an9 mutant line contained a more or less wild-type level of DFR mRNA, as can be deduced from the control hybridization with a CHS probe. The an3 mutant, blocked in the conversion of naringenin to dihydrokaempferol and thought to involve a lesion in the production of the flavanone-3-hydroxylase, showed a wild-type level of DFR mRNA.

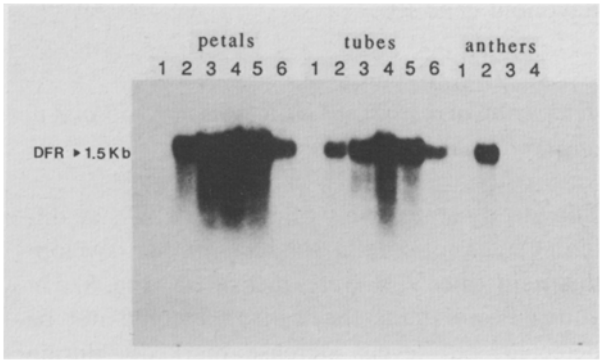

Fig. 6. Steady-state DFR mRNA levels during development of limb, tube and anthers of the line V30 Stage 1–6 represent size measures from small flower buds (4 mm) to mature flowers. The filter was washed under low stringency conditions (2× SSC; 1% SDS; 60 °C) after blotting and hybridization with the λDFR-A1 probe.

An overview of dihydroflavonol-accumulating mutants, their chromosomal position, steady-state levels of DFR mRNA and UDPglucose: 3-*O*-glucosyltransferase (UFGT) activity is presented in Table 3. The An6 locus is the only candidate for containing the structural DFR gene based on its chromosomal position. Besides, UFGT expression is normal in the an6 mutant, suggesting that the an6 mutant specifically influences DFR expression. For these reasons, we are presently performing an RFLP analysis for the DFR-A gene in a back-cross, segregating for An6. Definite proof that the An6 locus contains the DFR-A gene requires molecular complementation of the an6 mutant with the DFR-A gene.

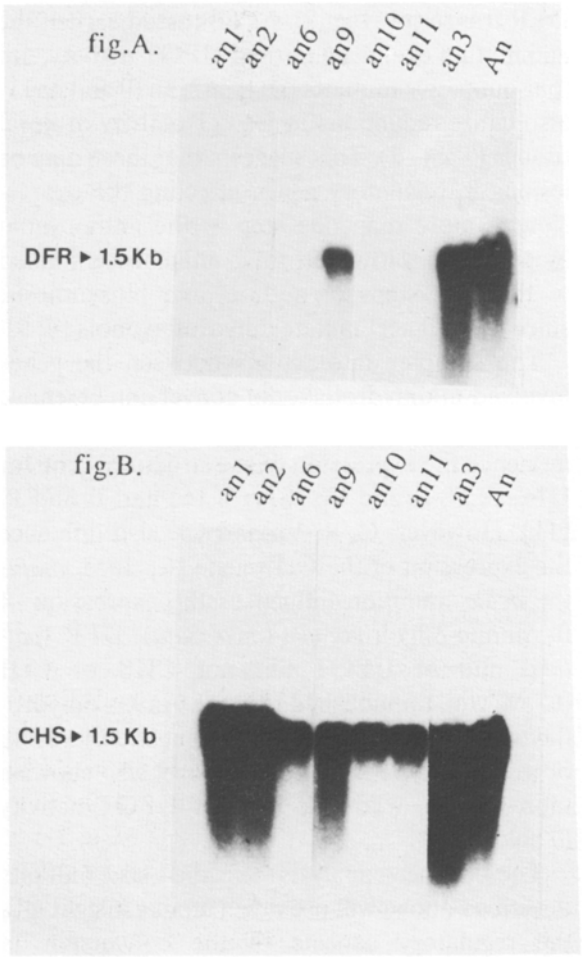

Fig. 7. Steady state DFR mRNA levels in maturing flowerbuds of dihydroflavonol accumulating mutants (an1, an2, an6, an9, an10, and 11), the earlier blocked an3 mutant and a wild-type (An) line. A: hybridized with λDFR-A1; B: the same filter stripped and hybridized with a CHS cDNA probe.

Discussion

Dihydroflavonol reductase genomic localization and gene structure in P. hybrida

Based on three types of data, we conclude that *P. hybrida* line V30 contains three DFR genes. First, Southern blot analysis of genomic V30 DNA showed the presence of three hybridizing bands (Fig. 4). Second, the genomic clones that were isolated from a V30 genomic library could be divided into three types, according to their restriction map (results not shown). RFLP analysis demonstrated the presence of DFR-A in chromosome IV, DFR-B in chromosome II and DFR-C in chromosome VI (Table 2).

Table 3. Steady-state DFR mRNA levels and relative UFGT activity in wild-type and dihydroflavonol-accumulating mutant lines

Locus	DFR mRNA[a]	UFGTact[b]	Chromosome
An[c]	100	100	
an3	100	90	IV
an1	n.d.[e]	7	VI
an2	n.d.[e]	3	VI
an6	n.d.[e]	91	IV
an9	90	95	I
an10	n.d.[e]	<10	I?
an11	n.d.[e]	<10	III

[a] Estimation by eye in % of the wild type.
[b] Calculated (wild type = 100%) from experiments, described by Gerats *et al.* [10, 12].
[c] An: wild-type line, dominant for all An genes mentioned here.
[e] n.d.: not detectable.

The genomic sequence of the *A. majus* gene (D. Barker, E. S. Coen and C. Martin, unpublished results) and of the DFR-A gene (results not shown) were compared to their respective cDNA sequences. The results indicated the presence of five introns in the reductase gene of both *A. majus* and *P. hybrida* (cf. Fig. 2). The DFR gene from *Z. mays* (A1) contains three introns [33], at the same positions as the first three introns in *A. majus* and *P. hybrida*.

The nucleotide sequence of the *A. majus* DFR cDNA contains a three-fold repetition of 75 nucleotides in the 3′ region (Fig. 2), which is not present in *P. hybrida* or *Z. mays*.

The structural locus encoding DFR

The an6 mutant exhibits no detectable DFR mRNA but has a wild type UFGT expression (Table 3), suggesting that it may represent the locus encoding the DFR gene expressed during

flower production. This hypothesis is currently being tested by phenotypic complementation of the an6 mutation with the DFR-A gene from line V30.

The occurrence of more than one genomic sequence homologous to the DFR cDNA is not unusual for *Petunia*. Multiple genes for CHS and CHI have been described earlier [22,34]. As a single DFR cDNA type was found it may be that the DFR-A gene is the only DFR gene active during flower development. DFR-B and DFR-C genes may be silent or may produce DFR-related transcripts with different catalytic properties, in different tissues or at different times.

DFR expression in flowers

Northern blot analysis of mRNA isolated from developmental stages of limb, tube and anther tissue of V30 indicated that DFR transcript levels increase dramatically during flower development in a manner similar to CHS and CHI mRNA [34]. Both limb and tube show a maximum steady-state level of DFR mRNA in flower buds of maximum length, just prior to unfolding of the petals. In contrast, anthers show a much earlier maximum (Fig. 6). This might represent a temporal shift rather than a developmental difference since anthers (and pistil) develop earlier than tube and limb. Meiosis normally is completed before the flower bud reaches a length of 3–4 mm.

Effect of mutations that suppress anthocyanin synthesis on DFR expression

Six *Petunia* mutants that suppress anthocyanin synthesis and accumulate dihydroflavonols, have been examined for their effects on DFR expression. These mutants might represent lesions in the biosynthetic enzymes involved in converting dihydroflavonols into anthocyanins, or lesions in proteins involved in anthocyanin transport or lesions in regulatory genes. The mutants were examined for DFR transcript levels. Surprisingly, five out of six mutants produced no detectable DFR transcript (Fig. 7). As discussed earlier, the an6 mutant exhibits a normal UFGT activity, but four out of five mutants (an1, an2, an10 and an11) also cause reductions in UFGT activity in floral tissues (Table 3). This suggests that these may be lesions in regulatory genes affecting the expression of more than one step in the anthocyanin biosynthesis. However, their influence is limited to the later steps of anthocyanin biosynthesis, since they all accumulate dihydroflavonols [4, 9].

The complex interactions between the genes involved in dihydroflavonol conversion resemble the situation in *Z. mays*. Six genes are known that influence the expression of the structural gene for UFGT: C, R and Vp [5], Clf [6] and B and Pl [11]. However, C, R, Vp and Clf also influence the expression of the CHS gene [6]. In *A. majus* the *delila* mutation influences the expression of flavanone-3-hydroxylase (*incolorata*), DFR (*pallida*) and of UFGT but not CHS or CHI (C. Martin, unpublished results). An9, a dihydroflavonol-accumulating mutant, that shows a more or less wild-type DFR mRNA level also shows a more or less wild-type level of UFGT activity (Table 3).

The further analysis of the six mutants described above will provide a unique insight into the regulatory aspects of the conversion of dihydroflavonols in anthocyanins in *Petunia hybrida*.

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References

1. Coe EH: Anthocyanin synthesis in maize – A gene action sequence construction. Am Nat 91: 381–385 (1957).
2. Dean C, van den Elzen P, Tamaki S, Dunsmuir P, Bedbrook J: Linkage and homology analysis divides the eight genes for the small subunit of petunia ribulose 1,5-bisphosphate carboxylase into three gene families. Proc Natl Acad Sci USA 82: 4964–4968 (1985).
3. Dellaporta SJ, Wood J, Hicks JB: A plant DNA mini-preparation, version two. Plant Mol Biol Rep 1: 19–21 (1983).
4. Doodeman M, Gerats AGM, Schram AW, de Vlaming P, Bianchi F: Genetic analysis of instability in *Petunia hybrida*. 2. Unstable mutations at different loci as the result of transpositions of the genetic element inserted at the *An1* locus. Theor Appl Genet 67: 357–366 (1984).
5. Dooner, HK, Nelson OE: Genetic control of UDP glucose: Flavonol 3-*O*-glucosyltransferase in the endosperm of maize. Biochem Genet 15: 509–519 (1977).
6. Dooner H: Coordinate genetic regulation of flavonoid biosynthetic enzymes in maize. Mol Gen Genet 189: 136–141 (1983).
7. Forkmann G, Ruhnau B: Distinct substrate specificity of dihydroflavonol-4-reductase from flowers of *Petunia hybrida*. Z Naturforsch, sect C Bioscience 42(9/10): 1146–1148 (1987).
8. Froemel S, de Vlaming P, Stolz G, Wiering H, Forkmann G, Schram AW: Genetic and biochemical studies on the conversion of flavanones to dihydroflavonols in flowers of *Petunia hybrida*. Theor Appl Genet 70: 561–568 (1985).
9. Gerats AGM, de Vlaming P, Doodeman M, Al B, Schram AW: Genetic control of the conversion of dihydroflavonols into flavonols and anthocyanins in flowers of *Petunia hybrida*. Planta 155: 364–368 (1982).
10. Gerats AGM, Wallroth M, Donker-Koopman W, Groot SPC, Schram AW: The genetic control of the enzyme UDPGlucose: 3-*O*-flavonoid glucosyltransferase in flowers of *Petunia hybrida*. Theor Appl Genet 65: 349–352 (1983).
11. Gerats AGM, Bussard J, Coe EH, Larson RL: Influence of *B* and *P1* on UDPG: flavonoid 3-*O*-glucosyltransferase in *Petunia hybrida*. Biochem Genet 22: 1161–1169 (1981).
12. Gerats AGM, Wallroth M, Vrijlandt E, Schram AW: On the genetic control of the enzyme UDPglucose: flavonoid 3-*O*-glucosyltransferase in *Petunia hybrida*. Biochem Genet 23: 591–598 (1985).
13. Gerats AGM: Mutable systems: their impact on flavonoid synthesis in *Petunia hybrida*. Ph.D. Thesis, University of Amsterdam (1985).
14. Gerats AGM, Veerman W, de Vlaming P, Wiering H, Cornu A, Farcy E, Maizonnier D: In: O'Brien SJ (ed) Genetic maps 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1987).
15. Harrison BJ, Stickland RG: Precursors and genetic control of pigmentation. 2. Genotype analysis of pigment controlling genes in acyanic phenotypes in *Antirrhinum majus*. Heredity 33: 112–115 (1974).
16. Heller W, Britsch L, Forkmann F, Grisebach H: Leucoanthocyanidins as intermediates in anthocyanin biosynthesis in flowers of *Matthiola incana* R. Br. Planta 163: 191–196 (1985).
17. Heller W, Forkmann G, Britsch L, Grisebach H.: Enzymatic reduction of (+)-dihydroflavonols to flavan-3,4-*cis*-diols with flower extracts from *Matthiola incana* and its role in anthocyanin biosynthesis. Planta 165: 284–287 (1985).
18. Henikoff S: Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28: 351–359 (1984).
19. Hrazdina G, in: Harbourne JB, Mabry TJ (eds) The flavonoids: Advances in Research, p. 170. Chapman and Hall, London.
20. Jonsson LMV, de Vlaming P, Wiering H, Aarsman MEG, Schram AW: Genetic control of anthocyanin-*O*-methyltransferase activity in flowers of *Petunia hybrida*. Theor Appl Genet 66: 349–355 (1983).
21. Koes RE, Spelt CE, Reif HJ, van den Elzen PJM, Veltkamp E, Mol JNM: Floral tissue of *Petunia hybrida* (V30) expresses only one member of the chalcone synthase multigene family. Nucl Acids Res 14: 5229–5239 (1986).
22. Koes RE, Spelt CE, Mol JNM, Gerats AGM: The chalcone synthase multigene family of *Petunia hybrida* (V30): sequence homology, chromosomal localization and evolutionary aspects. Plant Mol Biol 10: 159–169 (1987).
23. McLean M, Baird WV, Gerats AGM, Meagher RB: Determination of copy number and linkage relationships among five actin gene subfamilies in *Petunia hybrida*. Plant Mol Biol 11: 663–672 (1988).
24. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1982).
25. Martin C, Carpenter R, Sommer H, Saedler H, Coen R: Molecular analysis of instability in flower pigmentation of *Antirrhinum majus*, following isolation of the pallida locus by transposon tagging. EMBO J 4: 1625–1630 (1985).
26. Mol JNM, Schram AW, de Vlaming P, Gerats AGM, Kreuzaler F, Hahlbrock K, Reif HJ, Veltkamp E: Regulation of flavanoid gene expression in *Petunia hybrida*: description and partial characterization of a conditional mutant in chalcone synthase gene expression. Mol Gen Genet 192: 424–429 (1983).
27. O'Reilly C, Shepherd N, Pereira A, Schwarz-Sommer Z, Bertram I, Robertson DS, Peterson PA, Saedler H: Molecular cloning of the A1 locus of *Zea mays* using the transposable elements En and Mu1. EMBO J 4: 877–882 (1985).
28. Pichersky E, Bernatzky R, Tanksley SD, Breidenbach

RB, Kausch AP, Cashmore AR: Molecular characterization and genetic mapping of two clusters of genes encoding chlorophyll *a/b*-binding proteins in *Lycopersicon esculentum* (tomato). Gene 40: 247–258 (1985).
29. Rackwitz HR, Zehetner G, Frischauf AM, Lehrach H: Rapid restriction mapping of DNA cloned in lambda phage vectors. Gene 30: 195–200 (1984).
30. Reddy A, Britsch L, Salamini F, Saedler H, Rohde W: The A1 (anthocyanin-1) locus in *Zea mays* encodes dihydroquercetin reductase. Plant Sci 52: 7–13 (1987).
31. Reif HJ, Niesbach I, Deumling B, Saedler H: Cloning and analysis of two genes for chalcone synthase from *Petunia hybrida*. Mol Gen Genet 199: 208–215 (1985).
32. Schram AW, Jonsson LMV, Bennink GJH, in: Monographs on Theoretical and applied genetics. Springer-Verlag, Berlin (1984).
33. Schwarz-Sommer Z, Shepherd N, Tacke E, Gierl A, Rohde W, Leclercq L, Mattes M, Berndtgen R, Peterson PA, Saedler H: Influence of transposable elements on the structure and function of the A1 gene of *Zea mays*: EMBO J 6: 287–294 (1987).
34. van Tunen, AJ, Koes RE, Spelt CE, van der Krol AR, Stuitje AR, Mol JNM: Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: coordinate, light-regulated and differential expression of flavonoid genes. EMBO J 7: 1257–1263 (1988).
35. Wagner GJ: Content and vacuole/extravacuole distribution of neutral sugars, free amino acids, and anthocyanins in protoplasts. Plant Physiol 64: 88–93 (1979).
36. Wallroth M, Gerats AGM, Rogers SG, Fraley RT, Horsch RB: Chromosomal localization of foreign genes in *Petunia hybrida*. Mol Gen Genet 202: 6–15 (1986).
37. Wiering H, de Vlaming P, in: Monographs on Theoretical and Applied Genetics. Springer-Verlag, Berlin (1984).