Theor Appl Genet (1985) 70: 561–568

Genetic and biochemical studies on the conversion of flavanones to dihydroflavonols in flowers of *Petunia hybrida*

S. Froemel[1], P. de Vlaming[2], G. Stotz[1], H. Wiering[2], G. Forkmann[1] and A. W. Schram[2]

[1] Universität Tübingen, Institut für Biologie II, Lehrstuhl für Genetik, Auf der Morgenstelle 28, D-7400 Tübingen, Federal Republic of Germany

[2] Section Biosynthesis of Flavonoids, Department of Genetics and Plant Physiology, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands

Received November 4, 1984; Accepted February 28, 1985
Communicated by D. von Wettstein

Summary. Chemogenetic investigations and precursor experiments on flowers of *Petunia hybrida* suggest that recessive alleles of the gene *An3* block the biosynthetic pathway of flavonols and anthocyanins between the flavanone and dihydroflavonol step. In confirmation of this hypothesis, activity of the enzyme flavanone 3-hydroxylase, which catalyses the conversion of flavanones to dihydroflavonols, was readily demonstrated in enzyme preparations from flowers of lines with the dominant allele *An3*, whereas no or very low activity could be found in extracts from lines with recessive alleles (*an3an3*). A second genetic factor is described which clearly reduces the amount of flavonols in the flowers but not the amount of anthocyanins. Crossing experiments revealed that this factor represents a third allele of the *An3* gene. It is referred to as *an3-1*. As expected, a residual flavanone 3-hydroxylase activity of about 10% could be found in enzyme extracts from plants with the *an3-1* allele. The decreased level of dihydroflavonol formed under this condition is obviously still sufficient for anthocyanin formation but not for flavonol synthesis.

Similar to flavanone 3-hydroxylases from other plants, the enzyme of *Petunia* is a soluble enzyme and belongs according to its cofactor requirements to the 2-oxoglutarate-dependent dioxygenases. The residual flavanone 3-hydroxylase activity found in plants with the *an3-1* allele is identical to the activity extracted from *An3*-genotypes with regard to cofactors, substrate specificity and most of the inhibitors. The difference observed in the respective pH-optima and the genetic data suggest that the mutation providing the *an3-1* phenotype is localized in the structural gene for flavanone 3-hydroxylase.

Key words: Anthocyanin-Biosynthesis – Flavonoids – Flavanone 3-hydroxylase – Genetic Control – *Petunia hybrida*

Introduction

Many genes controlling the biosynthesis of anthocyanins and flavonols in *Petunia hybrida* have been described. Some of these genes control the synthesis and modification of the C15 skeleton. White flowering mutants, or those producing only low amounts of anthocyanins, are homozygous recessive for one of the *an* genes. Wiering (1974) and Kho et al. (1977) described the gene *An3* which is involved in one of the early steps of biosynthesis.

Flowers of plants homozygous recessive for the allele *an3* accumulate flavanones. The nature of the flavanones is determined by the genes *Ht1* and/or *Hf1* (Doodeman et al. 1982). Flower limbs of these mutants are able to synthesize anthocyanins upon feeding with dihydrokaempferol and other dihydroflavonols (Tabak et al. 1978) which indicates that the synthesis of dihydroflavonols is blocked in *an3/an3* plants. Furthermore, plants homozygous recessive for *an3* do not accumulate flavonols, even in the presence of the dominant allele of the flavonol gene *Fl*. Mutants homozygous recessive for *fl* accumulate small amounts of flavonols whereas mutants with a dominant allele accumulate 5–10 times the amount of kaempferol and quercetin. We suggest that *An3* is involved in the synthesis of precursors (dihydroflavonols) for flavonol and anthocyanin biosynthesis.

A second gene (*An7*) has been described (Wiering et al. 1979) which rise to intermediate levels of flavonol

in *Petunia* flowers in the presence of the dominant allele of *Fl*. It is suggested that the recessive allele *an7* represents a third allele of *An3*. In this paper we present data on the enzymatic reaction controlled by the different alleles of the locus *An3*.

Material and methods

Plant material

Information on the genotypes and phenotypes of the *an3an3* lines in our collection and lines used in crossings can be found in Table 1.

Description of the genes

An3-: synthesis of anthocyanins and flavonols in flower limb and flower tube
an3an3: no synthesis of flavonols, no or little synthesis of anthocyanins
An7-: synthesis of flavonols and anthocyanins in flower limb and flower tube (*An3-*)
an7an7: synthesis of flavonols in the flower limb decreased no or little synthesis of anthocyanins in the flower tube (*an3-1an3-1*)

The visible difference between the flowers of *An7-* and *an7an7* plants is most clear under a genetic background dominant for the genes *Fl, Hf1,Rt* and *An4;* the limbs of flowers of *an7an7* plants are more reddish than the limbs of *An7*-plants.

An4-: synthesis of anthocyanins in pollen and venation of flower limb and flower tube
an4an4: no synthesis of anthocyanins in pollen, decreased synthesis of anthocyanins in flower tube
Dw1-: plant form normal
dw1dw1: plant form dwarf with short internodes, small dark green leaves and small flowers
Fl-: synthesis of flavonols
flfl: no or little synthesis of flavonols
Ht1-: 3′-hydroxylation of flavonoids, synthesis of quercetin and cyanidin
ht1ht1: no synthesis of quercetin and little of cyanidin, accumulation of kaempferol
Hf1-: 3′,5′-hydroxylation of flavonoids, synthesis of delphinidin in flower limb and flower tube
hf1hf1: no or little synthesis of delphinidin in the flower limb and the flower tube
Rt-: synthesis of anthocyanidin-3-rutinoside
rtrt: anthocyanidin-3-glucoside present

The localisation of the genes in the biosynthetic pathway is shown in a proceeding paper (see Fig. 1, Stotz et al. 1985). The P values in the tables are at χ^2 level.

Chemicals and synthesis of substrates

Naringenin, dihydroquercetin, kaempferol, quercetin and myricetin were obtained from Roth (Karlsruhe, Federal Republic of Germany). Eriodictyol, 5,7,3′,4′,5′-pentahydroxyflavanone, dihydrokaempferol and dihydromyricetin were from our laboratory collection.

(2-^{14}C)Malonyl-CoA (2.22 GBq/mmol) was obtained from Amersham Buchler and diluted to 1.03 GBq/mmol with unlabelled material from Sigma. (4a,6,8-^{14}C)Naringenin and

Table 1. Genotypes and phenotypes of lines and plants used for the genetic and biochemical investigations

Line code or plant no.	Genotype[a]								Phenotype			
	An3-Locus	*An1*	*An4*	*Ht1*	*Hf1*	*Fl*	*Rt*	*Dw1*	Km[b]	Qu	My	Anthocyanins[c]
R3	+	+	+	+	–	–	–	+	21	53	0	+ + +
R12	+	+	+	–	–	–	–	–	150	10	0	+
R81 and R69	*an3-1*	+	+	–	–	–	–	+	0	0	0	+
M62	*an3-1*	+	–	–	–	–	+	+	50	0	0	+
V10	+	+	+	–	+	–	+	+	202	10	0	+ + +
V13 and V20	+	+	–	+	+	+	+	+	211	890	22	+ + +
V33	+	+	–	+	+	–	+	+	0	10	0	+ + +
V42	+	+	–	+	+	+	+	+	126	918	0	+ + +
W18	–	+	+	+	–	–	–	+	0	0	0	tr
W20	+	–	–	+	–	–	+	+	20	181	0	–
W37	–	+	+	–	–	–	–	+	0	10	0	–
W39	–	+	+	+	–	–	–	+	0	10	0	tr
W56	–	+		–	+	–	–	+	16	0	0	tr
W62	–	+	+	+	+	+	–	+	0	70	0	tr
W67	–	+	+	–	+	–	+	+	0	0	0	tr
G2108-82[d]	+	+	–	–	+/±	+/±	+	+	744	74	0	+ + +
G2108-55	*an3-1*	+	–	+/±	+/±	+/±	+	+	0	199	0	+ + +
G2108-122	*an3-1*	+	–	+/±	+/±	+/±	+	+	20	285	10	+ + +
G2096-7	*an3-1*	+	+/±	–	+/±	+/±	+/±	+	165	37	0	+ +
G2096-57	*an3-1*	+	–	+/±	+/±	+/±	+/±	+	0	250	0	+ +

[a] + = homozygous dominant; – = homozygous recessive; +/± = at least one allele dominant
[b] Relative amount of flavonol aglycone in integration units: Km = kaempferol; Qu = quercetin; My = myricetin
[c] + + + = high amount; + = small amount; tr = traces
[d] G2108 = F2 V13 × M62; G2096 = F2 M62 × W62

(4a,6,6-^{14}C)eriodictyol were prepared enzymatically (Forkmann et al. 1980). (4a,6,8-^{14}C) 5,7,3′,4′,5′-pentahydroxyflavanone was prepared enzymatically by incubation with a crude extract from purple bluish flower limbs of *Petunia hybrida* containing 3′,5′-hydroxylase activity (Stotz 1983).

Preparation of the crude extract

The preparation of the crude extract was performed according to Forkmann and Stotz (1981) with the exception that for 1 g of flower limbs, 5–10 ml buffer were used depending on enzyme activity.

Enzyme assay

The assay system contained in 100 µl total volume: 9.5 µmol Tris/HCl, pH 7.5; 0.056–0.156 µmol 2-mercaptoethanol; 0.045 or 0.06 µmol radioactive substrate (3.09 GBq/mmol); 2–25 µg protein; 0.5 µmol sodium ascorbate; 10 nmol ferrous sulfate and 25 nmol 2-oxoglutarate. Incubation was carried out for 2–60 min at 30 °C and was terminated by the addition of either 10 µg naringenin and dihydrokaempferol or eriodictyol and dihydroquercetin or pentahydroxyflavanone and dihydromyricetin in 10 µl methanol. The phenolics were extracted with ethylacetate and chromatographed on a cellulose plate with solvent system I or II. The plate was scanned for radioactivity. The radioactive zones were scraped off and counted in a scintillation counter.

Determination of pH optimum

The enzyme assays were carried out in mixtures of 175 µl 0.1 M Kpi buffer (between pH 5.0–8.5), 5 µl enzyme solution and 15 µl cofactor solution.

Analytical methods

Thin layer chromatography was performed on precoated cellulose plates (Schleicher and Schüll, FRG) with the following solvent systems (I) chloroform/acetic acid/water (10:9:1, v/v/v), (II) 15% acetic acid and (III) n-butanol/acetic acid/water (6:1:2, v/v/v).

For the identification of the reaction products radioactive zones were scraped off from the cellulose plates and eluted with methanol. After concentration, the eluates were co-chromatographed with authentic dihydroflavonols in the solvent systems mentioned above. Furthermore, the reaction products were used as substrates for enzymatic conversion to the respective flavonols with flower extracts of *Matthiola incana* (Spribille and Forkmann 1984).

Flavonoids were detected under UV-light and by treatment with ammonia fumes. Dihydroflavonols were also detected by treatment of the plates with zinc dust and subsequent spraying with 6M HCl (Barton 1968), and flavanones by reduction with borohydride and subsequent exposure to HCl fumes (Eigen et al. 1957).

Anthocyanins and flavonols in the flowers of the various genotypes investigated were analysed as described by Gerats et al. 1982. Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Results

Genetic studies

1 Localisation of the gene An3. Table 2 shows that there is linkage between the gene *An3* and the plant form gene *Dw1*. No cross-over type was found. The shortage of *dw1dw1* plants is due to the decreased viability of these plants.

In the search for a recombinant *an3/an3 dw1/dw1*, 65 flowering normal F2 plants were selfed. In the 4–8 leafed state, *dw1/dw1* plants can be identified. None of the 65 F2 plants, however, segregated for the gene *Dw1*, which indicates that the crossover percentage between *An3* and *Dw1* is less than 1%.

The gene *Dw1* is linked with the gene *Bl* which is located on chromosome IV (Maizonnier and Moessner 1979). Thus, the gene *An3* is situated on chromosome IV.

2 Linkage between An3 and An7. Several *an3/an3* lines with different genetic backgrounds for anthocyanin modificating genes had to be developed for the chemogenetical investigations and precursor experiments. The white flowering parents of these lines were isolated from F2 crosses between W37 and appropriate coloured lines. As no phenotypical expression of anthocyanin modificating genes in *an3/an3* plants is possible, the isolated plants had to be crossed with a coloured tester line. One of the tester lines used was the line R69, which is homozygous recessive for the gene *an7*. Ten randomly chosen *an3/an3* plants from one of the above mentioned F2 crosses were crossed with R69 and it was found that all the progeny plants were phenotypically *an7/an7*. No *an7/an7* plants were found among the coloured F2 plants tested (Table 3). Later we tested the line W37 which also appeared to be homogygous recessive for the gene *An7*. In the backcross W62 × (W67 × V33) (Table 3) no *an7/an7* plants were found; white flowering plants from this cross were not tested. From the data in Table 3 it can be concluded that there is at least a strong linkage between the genes *An3* and *An7*.

Both genes *An3* and *An7* are involved in flavonol synthesis. This together with the strong linkage suggests

Table 2. Cross R12 × W18;
F2 $\frac{an3Dw1}{An3dw1} \times \frac{an3Dw1}{An3dw1}$; F3 $\frac{an3Dw1}{an3\ -} \times \frac{an3Dw1}{an3\ -}$

Genotype and no. of progeny			
	Dw1 –	*dw1/dw1*	
An3 –	519	218	
an3/an3	259	0	
Segregation	*An3*	737:259	$P_{3:1} = 0.81$
	Dw1	778:218	$P_{3:1} = 0.01$
Linkage *An3–Dw1* $P_{2\times2} = < 0.001$			

No. of F3's: 65; total number of plants 9531 (seedlings)
All plants normal, no dwarfs
Cross-over percentage < 1

Table 3. Cross W37 × V20; F2 $\frac{an3}{An3}\ \frac{an7}{An7} \times \frac{an3}{An3}\ \frac{an7}{An7}$
Cross W62 × (W67 × V33);
Backcross $\frac{an3}{an3}\ \frac{an7}{an7} \times \frac{an3}{An3}\ \frac{an7}{An7}$

Expected segregating types			Results	
Genotype	Phenotype		No. of plants	
			F2	Backcross
An3/–An7/–	coloured limb coloured tube		787	779
An3/–an7/an7	coloured limb tube not coloured		0	0
an3/an3 an7/an7	white		238[a]	664
Segregation *An3*	F2:	787:238	$P_{3:1} = 0.24$	
	Backcross:	779:664	$P_{1:1} = < 0.001$	

[a] 10 plants tested × R69: all *an7/an7*

that the recessive allele *an7* is an allele of the gene *An3*, intermediate between the alleles *An3* and *an3*.

In Table 4 two crosses are given in which the three alleles *An3*, *an3-1* and *an3* are involved. From these results we assume that *An7* is allelic to *An3;* therefore the recessive allele *an7* is called *an3-1*.

3 Expression of An3 and an3-1. Table 5 shows the influence of *an3-1* on flavonol and anthocyanin concentration in flowers. The effect on flavonol concentration was investigated in plants containing at least one dominant Fl allele (plants homozygous recessive for *fl* accumulate only low levels of flavonol; Gerats et al. 1982), whereas the effect on anthocyanin concentration was studied in mutants homozygous recessive for *fl* (plants containing at least one dominant *Fl* and *Htl* allele and being homozygous recessive for *hfl* accumulate lower levels of anthocyanins; Gerats et al 1982). *an3-1* had no observed effect on anthocyanin concentration. Flavonol concen-

Table 4. Cross M62 × W62; F2 $\frac{an3\text{-}1}{an3} \times \frac{an3\text{-}1}{an3}$
Cross (R81 × V42) × R81; Backcross $\frac{an3\text{-}1}{An3} \times \frac{an3\text{-}1}{an3\text{-}1}$

Genotype	Phenotype	No. of plants F2		Backcross	
		Expected	Found	Expected	Found
An3/an3 *An3/an3-1*	coloured limb coloured tube	0	0	144	140
an3-1/an3-1 } *an3-1/an3* }	coloured limb tube not coloured	68	69	144	148
an3/an3	white	23	22	0	0

Table 5. Effect of *an3-1* on flavonol and on anthocyanin concentration in purple (*Hfl-Rt-*) flowers of *Petunia hybrida*

Genotype		cross (R81 × V42) × R81	Flavonol[b,c]			cross (R81 × V10) × R81	Anthocyanin[b]
		No. of plants[a]	Km	Qu	Total	No. of plants[d]	
An3/–	*Htl/htl*	33	28	198	226	14	0.77
an3-1/an3-1	*Htl/htl*	32	1	127	128	14	0.68
an3/an3[e]	*Htl/–*		0	25	25	–	0 or trace
An3/–	*htl/htl*	32	287	7	294	14	0.75
an3-1/an3-1	*htl/htl*	31	72	6	78	14	0.74
an3/an3[e]	*htl/htl*		35	0	35	–	0 or trace

[a] All plants contain at least one dominant Fl allele
[b] Relative quantities, mean values
[c] According to the students test the data for flavonol concentrations between the different genotypes differ significantly
[d] All plants are homozygous recessive for fl
[e] *an3/an3* plants for comparison

trations, however, were decreased. It should be noted that in *an3-1/an3-1* plants, kaempferol synthesis is relatively more inhibited than quercetin synthesis.

Biochemical studies

The conversion of flavanones to dihydroflavonols is catalysed by the enzyme flavanone 3-hydroxylase. Therefore, the demonstration of this enzyme activity in flower extracts of *Petunia* should definitely prove that the different *An3* alleles affect this step in anthocyanin biosynthesis.

1 Demonstration of flavanone 3-hydroxylase activity. When a crude extract prepared from flowers of line R3 (genotype *An3/An3*) was incubated with (^{14}C)naringenin and the cofactors for flavanone 3-hydroxylase, 2-oxoglutarate, ascorbate and Fe^{2+}, the radiochromatogram of the reaction mixture in solvent system I showed one reaction product (Fig. 1a). This product was identified as dihydrokaempferol by co-chromatography with an authentic sample in three different solvent systems and by its enzymatic conversion to kaempferol (Spribille and Forkmann 1984).

Incubations with crude extracts prepared from flowers of other lines with the dominant *An3* allele also led to the formation of dihydrokaempferol. Furthermore, when enzyme preparations from lines with dominant alleles of both the *An3* and the *Fl* locus were used as enzyme source, the formation of kaempferol, in addition to that of dihydrokaempferol, was observed (Froemel, unpublished results). Of all the lines investigated, line R3 showed the highest flavanone 3-hydroxylase activity. Moreover, the conversion of dihydroflavonols to flavonols is blocked in this line because recessive *fl* alleles are present (Froemel, unpublished results). Line R3 was therefore used for a further characterisation of the 3-hydroxylase reaction.

2 Genetic control of enzyme activity. All lines with the dominant *An3* allele exhibited high flavanone 3-hydroxylase activity, although appreciable differences were observed in their specific activity (Table 6). These differences are mainly due to different protein amounts of the crude extracts of the lines. They are possibly also caused by the influence of the genetic background which is not isogenic in the lines investigated.

In contrast to lines having the dominant *An3* allele (Fig. 1a), the lines with recessive alleles (*an3/an3*) completely lack flavanone 3-hydroxylase activity or show only a very low enzyme activity (Fig. 1c, Table 6). The latter observation is in good agreement with the fact that sometimes a very pale colouration of the flowers by anthocyanins occurs in these mutants.

The plants with the *an3-1* allele were selected among F_2 plants from two different crosses (see Table 1). When

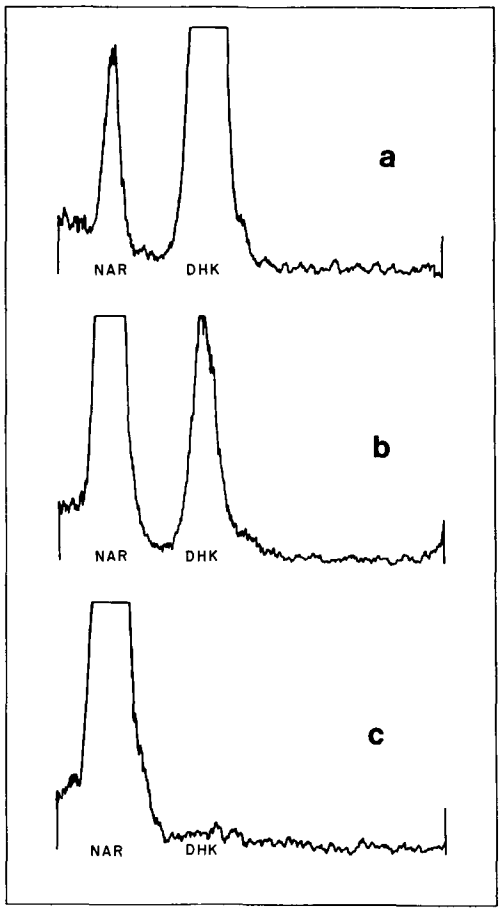

Fig. 1a–c. Radioscan of TLC on cellulose with solvent system I from incubations of (^{14}C) naringenin with enzyme preparations from different genotypes of *Petunia hybrida* in the presence of 2-oxoglutarate, ascorbate and Fe^{2+}. **a** line R3 (*An3An3*); **b** plant G2108-122 (*an3-1an3-1*); **c** line W39 (*an3an3*). NAR = naringenin; DHK = dihydrokaempferol

Table 6. Activity of flavanone 3-hydroxylase in flower extracts of the different genotypes investigated

Line code or plant no.	Allele at the *An3*-locus	Specific activity (μkat/kg protein)	Relative activity (%)
R3	*An3/An3*	13.38	
V13	*An3 An3*	7.70	
W20	*An3/An3*	6.63	
W37	*an3/an3*	0	
W39	*an3/an3*	0	
W56	*an3/an3*	0	
W62	*an3/an3*	< 0.3	
G2108-82	*An3/An3*	9.75	100
G2108-55	*an3-1/an3-1*	1.63	16.7
G2108-122	*an3-1/an3-1*	0.44	4.5
G2096-7	*an3-1/*±	0.82	8.4
G2096-57	*an3-1/*±	0.87	8.9

± = *an3-1* or *an3*

enzyme preparations from flowers of these plants were used as enzyme sources, the formation of dihydrokaempferol could be observed (Fig. 1 b). However, this enzyme activity was found to be considerably lower than that found in flower extracts prepared from genotypes having the *An3* allele (Fig. 1 a, b; Table 6). Thus, the enzyme activity in flower extracts of *an3-1* plants was found to be on an average less than 10% of the enzyme activity extracted from flowers of a comparable *An3* plant (Table 6) selected from one of the F2 progenies (see Table 1).

3 Dependence of enzyme activity on cofactors. When the enzyme assays were carried out without the three cofactors, 2-oxoglutarate, ascorbate and Fe^{2+}, a considerably lower dihydrokaempferol formation was observed, both for the crude extracts from lines with the *An3* allele and from plants with the *an3-1* allele (Table 7). Moreover, enzyme preparations from which low molecular weight substances had been removed by gel filtration on Sephadex G-50 were found to be completely inactive (Table 7). Addition of a combination of the three cofactors to such an incubation mixture led to a restoration of flavanone 3-hydroxylase activity. A pronounced effect was also found upon addition of only 2-oxoglutarate (only for line R3) or in combination with either ascorbate or Fe^{2+} (Table 7).

4 Substrate specificity. Eriodictyol and 5,7,3′,4′, 5′-pentahydroxyflavanone derivatives have been demonstrated in flowers of *Petunia hybrida* in addition to naringenin (Doodeman et al. 1982). Therefore, the question of whether these flavanones with higher hydroxylation of the B-ring are also substrates for 3-hydroxylation, was investigated. Enzyme preparations from lines with the *An3* allele as well as plants with the *an3-1* allele were found to catalyse hydroxylation of eriodictyol and 5,7,3′,4′,5′-pentahydroxyflavanone in the 3-position to dihydroquercetin and dihydromyricetin, respectively. Both dihydroflavonols were identified as described above. In comparison to dihydrokaempferol formation using naringenin as the substrate the rate of formation of dihydroquercetin from eriodictyol was only about 50% and the rate of formation of dihydromyricetin from 5,7,3′,4′,5′-pentahydroxyflavanone was even less than 10%.

5 Further characterisation of flavanone 3-hydroxylase. With enzyme preparations from flowers of line R3 (*An3/An3*), the synthesis of dihydrokaempferol was linear with protein concentration up to about 10 µg and with time for about 10 min. As expected from the considerably lower enzyme activity, in incubations with flower extracts from plants with the *an3-1* allele the reaction was found to be proportional with added enzyme extract up to 30 µg protein per assay; with 20 µg protein a linearity with time for at least 40 min was observed.

Surprisingly, the enzymes extracted from line R3 and from *an3-1* plants also differed in their pH-optimum. Thus, the highest conversion of naringenin to dihydrokaempferol was found around pH 6.0 when flower extracts from line R3 were used as the enzyme source whereas the enzyme preparations from plants with the *an3-1* allele exhibited maximal activity between pH 6.5 and pH 7.0.

Independently of the enzyme source used, the 3-hydroxylase reaction was strongly inhibited by KCN,

Table 7. Cofactor requirement of flavanone 3-hydroxylases from flower of line R3 (*An3An3*) and of plant G2108–122 (*an3-1an3-1*)

Enzyme source	Cofactors added			cpm in dihydrokaempferol	
	2-Oxo-glutarate	Ascorbate	Fe^{2+}	Line R3	Plant G2108–122[b]
Crude extract	+	+	+	1,839	1,377
	–	–	–	318	109
Crude extract	–	–	–	0	0
after gel filtration	+	–	–	1,081	116
(Sephadex G-50)	–	+	–	148	16
	–	–	+	217	197
	+	+	–	1,540	773
	+	–	+	2,422	1,200
	–	+	+	292	353
	+	+	+	3,270	1,560

[a] Reaction product formed with 5 µg protein after 5 min of incubation
[b] Reaction product formed with 20 µg protein after 30 min of incubation

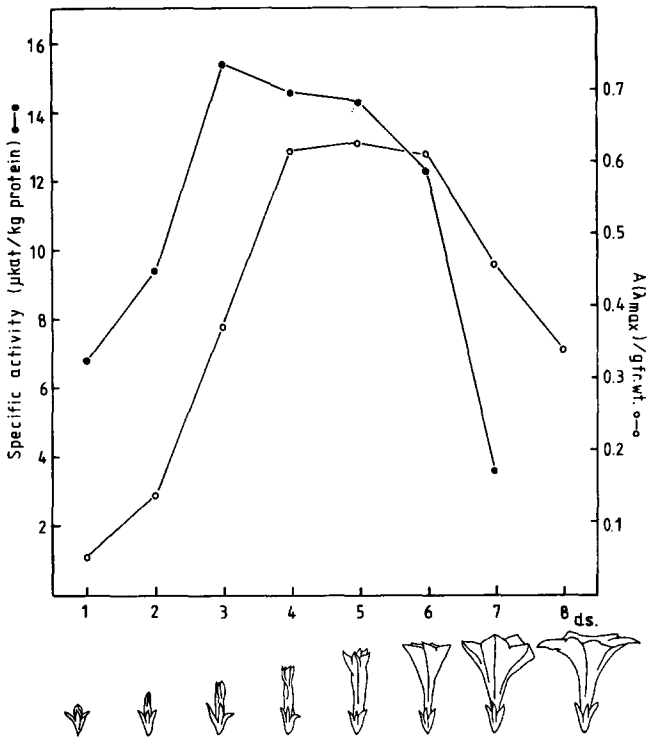

Fig. 2. The course of anthocyanin content and flavanone 3-hydroxylase activity during bud and flower development of *Petunia*. d.s. = developmental stages

Table 8. Effect of inhibitors on 3-hydroxylation of naringenin

Additions	3-Hydroxylation activity (%)	
	Line R3 (*An3An3*)	Plant G2108 (*an3-1an3-1*)
None	100.0	100.0
2 mM KCN	13.5	49.9
1 mM EDTA	50.1	10.9
4 mM EDTA	21.3	8.8
2 mM Diethyldithiocarbamate	31.7	0.6
4 mM Diethyldithiocarbamate	12.3	0
0.5 mM Diethylpyrocarbonate	109.5	126.9
4 mM Diethylpyrocarbonate	107.5	160.4
0.1 mM Chloromercuribenzoate	108.3	103.4

EDTA and diethyldithiocarbamate whereas p-chloromercuribenzoate had a slightly stimulating effect on enzyme activity (Table 8). A slight stimulation was also found in the presence of diethylpyrocarbonate in incubations with the enzyme from flowers with the dominant *An3* allele, whereas an unexpected high stimulation by this compound was observed in incubations with the enzyme extracted from *an3-1* plants (Table 8).

6 Dependence of enzyme activity on bud and flower development. The course of flavanone 3-hydroxylase activity as well as of anthocyanin accumulation in the flowers was studied during bud and flower development. Morphological criteria were used to divide the developmental process into eight significantly different stages (Fig. 2).

The amount of anthocyanins is very low in buds of stage 1 but increases rapidly up to a maximum value in the following three bud stages. After a period of nearly constant anthocyanin content, a rapid decline in the amount of anthocyanin per gram fresh weight was found in the completely opened flowers (Fig. 2). No decrease in total anthocyanin per flower was observed. The flavanone 3-hydroxylase activity increases rapdily from a moderate level in stage 1 to a maximum in stage 3. In enzyme preparations of the following stages, the enzyme activity decreases at first slightly, then progressively faster (Fig. 2). Corresponding investigations on different developmental stages of *an3-1* plants led to essentially similar results.

Discussion

The enzyme flavanone 3-hydroxylase, which catalyses the conversion of flavanones to dihydroflavonols, has been demonstrated in flower extracts of several plants (Forkmann et al. 1980; Forkmann and Stotz 1981; Forkmann and Stotz 1984). We have now been able to demonstrate this enzyme activity in flower extracts of *Petunia hybrida* and to investigate the 3-hydroxylase reaction in different genotypes affecting flavonoid synthesis in the flower.

In confirmation of earlier work on *Petunia* (Kho et al. 1977; Tabak et al. 1978; Doodeman et al. 1982) and of the genetic data presented in this paper, enzyme activity for 3-hydroxylation of flavanones was present in enzyme preparations from genotypes with the dominant allele *An3*, whereas no or very low enzyme activity was found in flower extracts from genotypes with recessive alleles of this gene. Thus, the interruption of the flavonoid pathway caused by recessive alleles of the gene *An3* concerns the step flavanone → dihydroflavonol and the gene *An3* actually controls the activity of flavanone 3-hydroxylase. Similar to the flavanone 3-hydroxylase from other plants (Forkmann et al. 1980; Forkmann and Stotz 1981; Forkmann and Stotz 1984; Britsch et al. 1981) the enzyme of *Petunia* is a soluble enzyme and belongs, according to its cofactor requirements, to the 2-oxoglutarate-dependent dioxygenases (Abbott and Udenfriend 1974).

Moreover, a similar effect of inhibitors on enzyme activity was observed. The pH at which maximal activity is exhibited is different from those of the 3-hydroxylases extracted from other plants. Nevertheless, most of the experiments described in this paper were done at neutral pH. We have chosen this condition in order to be able to compare our results with the results obtained in earlier studies. Under the experimental conditions described in Material and methods, the highest

3-hydroxylase activity was detected with naringenin as a substrate. In comparison to naringenin, only 50% of the enzyme activity were observed with eriodictyol as substrate, and with 5,7,3′,4′,5′-pentahydroxyflavanone as substrate only a minor activity of 10% was found. At present there are no unequivocal data available in the literature which could provide evidence whether 3′-hydroxylation occurs before or after 3-hydroxylation. The results presented in this paper might be taken to mean that 3′-hydroxylation takes places after 3-hydroxylation.

A correlation was found between the presence of flavanone 3-hydroxylase activity and anthocyanin content during bud and flower development. A similar relationship was also observed for other enzymes involved in the synthesis of flavonoids in *Petunia* (Stotz et al. 1985).

In flower extracts from plants with the *an3-1* allele a residual 3-hydroxylase activity of about 10% was found. This result provides evidence that the *an3-1* allele, as expected from the genetic data, is also involved in the conversion of flavanones to dihydroflavonols. The residual enzyme activity behaved identically to the activity extracted from *An3*-genotypes with regard to cofactors, substrate specificity and most of the inhibitors. We observed, however, a difference in diethylpyrocarbonate sensitivity and in the pH value at which maximal activity was observed. This could indicate that the enzyme protein synthesized in *an3-1* genotypes is structurally different from the enzyme protein of *An3* genotypes. Furthermore, the genetic data indicate that *an3* and *an3-1* represent alleles of the same locus. The mutation giving rise to the *an3-1* phenotype might therefore be localized in the structural gene for flavanone 3-hydroxylase.

It is interesting to see that the *an3-1* mutation does not effect anthocyanin content but has a dramatic effect on flavonol content. This observation can be rationalized in two ways. The most attractive explanation is that a decreased level of 3-hydroxylase gives rise to a low steady state concentration of dihydroflavonols. This decreased amount of dihydroflavonol could still be saturating for anthocyanin formation but not for flavonol synthesis. A second possibility is a difference in the subcellular compartimentation and/or chanelling between flavonol and anthocyanin in the last steps of their formation.

Acknowledgement. These investigations were supported by a grant from the Deutsche Forschungsgemeinschaft.

References

Abbott MT, Udenfriend S (1974) α-ketoglutarate-coupled dioxygenases. In: Hayaishi O (ed) Molecular mechanisms of oxygen activitation. Academic Press, New York London, pp 167–214

Barton GM (1968) Detection of 3-hydroxylflavanones on papergrams and thin-layer plates. J Chromatogr 34:562

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Britsch L, Heller W, Grisebach H (1981) Conversion of flavanone to flavone, dihydroflavonol and flavonol with an enzyme system from cell cultures of parsley. Z Naturforsch 36:742–750

Doodeman M, Tabak AJH, Schram AW, Bennink GJH (1982) Hydroxylation of cinnamic acids and flavonoids during biosynthesis of anthocyanins in *Petunia hybrida* hort. Planta 154:546–549

Eigen E, Blitz M, Gunsberg E (1957) The detection of some naturally occuring flavanone compounds on paper chromatograms. Arch Biochem Biophys 68:501

Forkmann G, Heller W, Grisebach H (1980) Anthocyanin biosynthesis in flowers of *Matthiola incana.* Flavanone 3- and flavonoid 3′-hydroxylases. Z Naturforsch 35:691–695

Forkmann G, Stotz G (1981) Genetic control of flavanone 3-hydroxylase activity and flavonoid 3′-hydroxylase activity in *Antirrhinum majus* (Snapdragon). Z Naturforsch 36:411–416

Forkmann G, Stotz G (1984) Selection and characterisation of flavanone 3-hydroxylase mutants of *Dahlia, Streptocarpus, Verbena* and *Zinnia.* Planta 161:261–265

Gerats AGM, de Vlaming P, Doodeman M, Al B, Schram AW (1982) Genetic control of the conversion of dihydroflavonols into flavonols and anthocyanins in flowers of *Petunia hybrida.* Planta 155:364–268

Kho KFF, Bolsman-Louwen AC, Vuik JC, Bennink GJH (1977) Anthocyanin synthesis in a white flowering mutant of *Petunia hybrida.* Planta 135:109–118

Maizonnier D, Moessner A (1979) Localisation of the linkage groups on the seven chromosome of the *Petunia hybrida* genome. Genetics 51:143–148

Spribille R, Forkmann G (1984) Conversion of dihydroflavonols to flavonols with enzyme extracts from flower buds of *Matthiola incana* R. Br. Z Naturforsch 39:714–719

Stotz G (1983) Enzymologie und Genetik der Oxidationsreaktionen in der Flavonoid-Biosynthese höherer Pflanzen. Ph D Thesis, Universität Tübingen (FRG)

Stotz G, de Vlaming P, Wiering H, Schram AW, Forkman G (1985) Genetic and biochemical studies on flavonoid 3′-hydroxylation in flowers of *Petunia hybrida.* Theor Appl Genet 70:300–305

Tabak AJH, Meyer H, Bennink GJH (1978) Modification of the B-ring during flavonoid synthesis in *Petunia hybrida:* introduction of the 3′-hydroxyl group regulated by the gene *Ht1.* Planta 139:67–71

Wiering H (1974) Genetics of flower colour in *Petunia hybrida hort.* Genen Phaenen 17:117–134

Wiering H, de Vlaming P, Cornu A, Maizonnier D (1979) *Petunia* genetics. 1. List of genes. Ann Amelior Plant 29:611–622