Genetic and Biochemical Studies on the Conversion of Dihydroflavonols to Flavonols in Flowers of *Petunia hybrida*

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

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Soluble enzyme preparations from flower buds of *Petunia hybrida* catalyzed the conversion of dihydroflavonols to flavonols. Dihydrokaempferol and dihydroquercetin were readily converted to the respective flavonols, whereas dihydromyricetin was a poor substrate. The reaction required 2-oxoglutarate, ascorbate and Fe^{2+} as cofactors and had a pH optimum at about 6.5. In the presence of the dominant allele *Fl*, high enzyme activity for flavonol formation was found, whereas in enzyme preparations from flower buds of recessive genotypes (*fl/fl*) only low enzyme activity could be observed. A substantial correlation was found between enzyme activity for flavonol formation and the flavonol content of buds and flowers during development.

Introduction

In the late sixties, Grisebach and co-workers had already shown by feeding experiments with radioactive precursors that dihydroflavonols are readily incorporated into flavonols in *Pisum sativum* [1] and *Datisca cannabina* [2]. *In vitro* conversion of dihydroflavonols to flavonols, however, was first observed in 1981 with enzyme preparations of parsley cell suspension cultures [3]. The reaction was found to be catalysed by a soluble enzyme which requires 2-oxoglutarate, ascorbate and Fe^{2+} ions as cofactor. Recently, a soluble enzyme catalysing the reaction dihydroflavonol → flavonol with the cofactors mentioned above was also observed in enzyme preparations from small flower buds of *Matthiola incana* [4]. Although many genes are known to control single steps in flavonoid biosynthesis in this plant [5], a gene concerning the step dihydroflavonol → flavonol has not been identified. Therefore, a correlation between genotype and enzyme activity for flavonol formation which definitely proves the relevance of the *in vitro* measured enzyme activity for the formation of flavonols *in vivo* could not be established as yet.

In flowers of *Petunia hybrida,* however, the gene *Fl* seems to control the formation of flavonols [6, 7]. In mutants with recessive alleles (*fl/fl*) flavonol synthesis is greatly reduced, whereas in flowers with the dominant allele 5 to 10 times the amount of flavonol is accumulated.

The presence of much flavonol has a distinct bluing effect on the flower colour. Furthermore, mutants homozygous recessive for *fl* and, in addition, for one of the genes *An1, An2, An6* or *An9*, which block anthocyanin formation late in biosynthesis, accumulate dihydroflavonols. But similar mutants with the dominant allele of *Fl* accumulate flavonols instead of dihydroflavonols (Fig. 2).

We now report on the enzymatic conversion of dihydroflavonols to flavonols with enzyme preparations from flowers of *Petunia* and on the first successful correlation between genotype and enzyme activity for flavonol formation.

Material and Methods

Plant material

For the genetic experiments two inbred lines were used:

Vu6: flower colour grey 2 reddish HCC nr. 0023/1; genotype: *fl/fl*

V42: flower colour purple bluish HCC nr. 38; genotype: *Fl/Fl*

Plants were visually divided in flower colour classes [6]. Unfortunately, the two lines Vu6 and V42 were not available for the biochemical investigations. Therefore, the latter studies were performed on a couple of comparable lines with dominant (lines

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V13, V23, V28, W4 and W42) or recessive (lines R3, R4, R27, V12, V33, W39 and W78) alleles at the locus *Fl.* Information on the genotypes and phenotypes of these lines can be found in Table III. The action of the genes in the biosynthetic pathway is shown in Fig. 2.

The plant material was cultivated in a greenhouse.

Authentic flavonoids

Naringenin, dihydroquercetin, kaempferol, quercetin and myricetin were obtained from Roth (Karlsruhe, Germany). Dihydrokaempferol and dihydromyricetin were from our labarotory collection.

Labelled substrates

[2-^{14}C]Malonyl-CoA (2.22 GBq/mmol) was obtained from Amersham Buchler (Braunschweig, Germany) and diluted to 1.03 GBq/mmol with unlabelled material from Sigma. [4a,6,8-^{14}C]Naringenin, [4a,6,8-^{14}C]dihydrokaempferol, [4a,6,8-^{14}C]dihydroquercetin and [4a,6,8-^{14}C]dihydromyricetin (all 3.09 GBq/mmol) were prepared enzymatically using enzyme preparations from parsley [3, 8] and from *Petunia hybrida* cv. "Red Titan" and "Blue Titan" [9, 10].

Preparation of crude extract

All steps were carried out at 4 °C. 1.0 g buds or flowers were homogenized in a prechilled mortar together with 0.5 g Dowex 1X2, 0.5 g quartz sand and 3 ml 0.1 M potassium phosphate buffer, pH 7.0 with 28 mM 2-mercaptoethanol. The homogenate was centrifuged twice for 5 min each at about 10,000 × *g*. The supernatant of the second centrifugation served either directly as enzyme source at pH 7.0 or was subjected to gel filtration on Sephadex G-50 in order to remove low molecular weight substances and to change the pH value of the preparation from 7.0 to 6.5.

Standard enzyme assay

The incubation mixture contained in a total volume of 200 µl: 20 µmol potassium phosphate (pH 7.0 or 6.5), 0.28 µmol 2-mercaptoethanol, 0.04 nmol radioactive substrate (dihydrokaempferol, dihydroquercetin or dihydromyricetin), 50 nmol 2-oxoglutarate, 1 µmol ascorbate, 10 nmol ferrous sulfate and 10 µl enzyme extract (about 40 µg protein). Incubation was carried out for 5 min at 30 °C. The mixture was immediately extracted twice with ethyl acetate (80 µl and 50 µl) and chromatographed on a cellulose plate with solvent system 1. Radioactivity was localized by scanning the plate (TLC Analyzer, Berthold Wildbad, Germany) and enzyme activity was determined by integration of the peak areas of the dihydroflavonol used as substrate and the respective flavonol formed. The radioactive zones were also scraped off and counted in a scintillation counter.

Dependence of the reaction on pH

The enzyme assays were carried out in mixtures of 165 µl 0.1 M potassium phosphate buffer (between pH 5.5 and 8.5), 5 µl crude extract and 30 µl aqueous cofactor solution.

Analytical methods

Protein was determined according to Bradford [11] using bovine serum albumin as a standard.

Thin layer chromatography was performed on precoated cellulose plates (Schleicher & Schüll, Dassel, Germany) in: 1) 30% acetic acid; 2) chloroform/acetic acid/water (10:9:1), v/v/v; 3) acetic acid/HCL/water (30:3:10), v/v/v.

The reaction products (flavonols) were identified by chromatography with authentic samples in the 3 solvent systems mentioned above.

Flavonoids were detected on chromatograms under UV light and after fuming with ammonia or by spraying the plates with 1.0% aqueous fast-blue salt B and subsequent exposure to ammonia vapors. Dihydroflavonols were also demonstrated by treatment of the plates with zinc dust followed by spraying with 6 M HCL [12].

The flavonol content of buds and flowers during development was estimated as described [13] with subsequent acid hydrolysis of the extracts.

Flavonol extracts in the genetic experiment were obtained by hydrolyzing 5 pieces of 1 cm^2 of one flower limb in 1 ml 2 M HCL during 15 min at 100 °C. 0.5 ml of this extract was extracted with 50 µl isoamylalcohol. 4 µl of the upperphase was chromatographed on Whatman 1 paper with Forestal as eluens. Flavonol concentration was measured by comparing the spots under UV light visually with spots in a concentration range between 0.25 and 5.00 microgram of quercetin on a developed chromatogram.

Results

Genetic studies

In Fig. 1, the F2 and B1 of the cross Vu6 × V42, the segregations in flower colour classes and their relation to flavonol content are presented.

A monofactorial segregation in plants with much flavonol (1.5–5 microgram/sample) and plants with traces of flavonol (0–1.25 microgram/sample), corresponding with the colour classes bluish type (genotype *Fl/.*) and reddish type (genotype *fl/fl*), respectively, is found.

The deviation of the 3:1 ratio in the F2 is probably due to certation.

Biochemical studies

Crude extracts were prepared from early flower stages (stages 2 to 4), which have been found previously to exhibit maximum activity of flavanone 3-hydroxylase [15] and flavonoid 3′-hydroxylase [14]. These enzymes provide the different hydroxylated dihydroflavonols which are expected to be the substrates for flavonol formation.

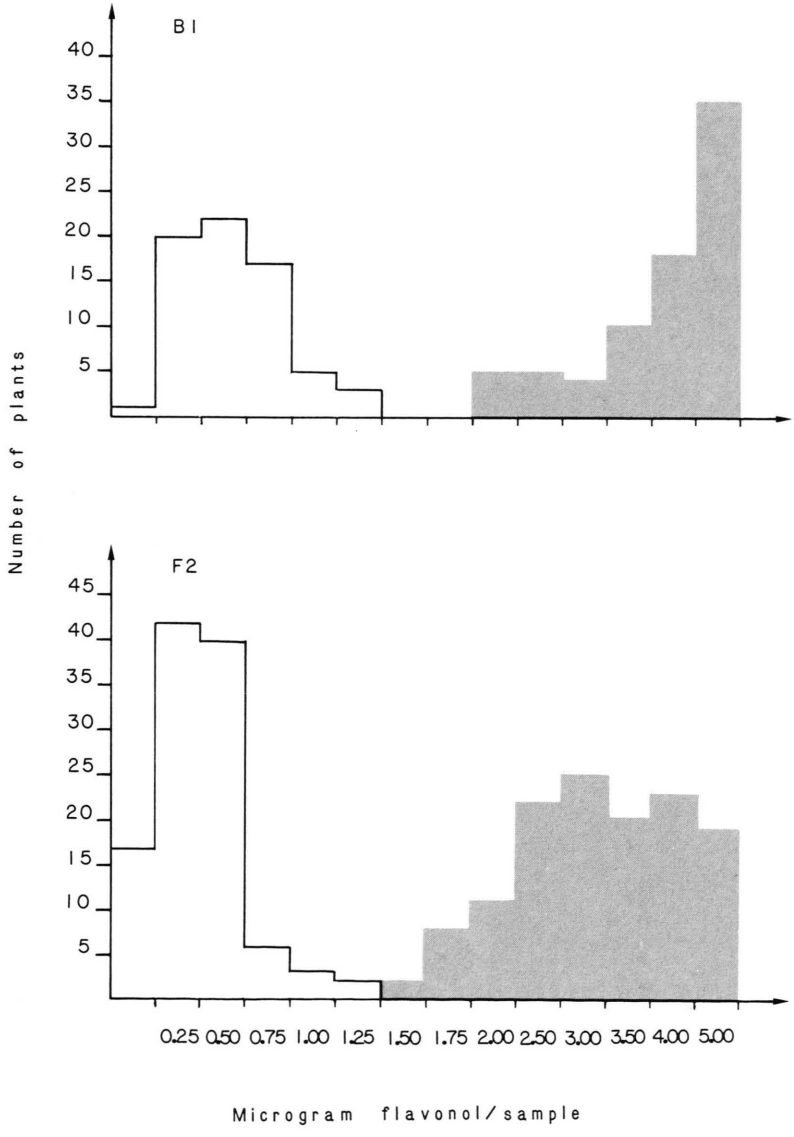

Fig. 1. Flavonol content of flower limbs of plants from the F2 and B1 of the cross Vu6 × V42.
In the F2 a 333:246 segregation for *Fl:fl* was found ($\chi^2_{3:1} = 94.43$ $P = < 0.001$); in the B1 a 78:79 segregation ($\chi^2_{1:1}$: 0.006 $P = 0.94$); not all plants were analyzed.
□ Reddish types (*fl/fl*);
■ bluish types (*Fl/.*).

Incubation of enzyme preparations from lines with the dominant allele *Fl* with [^{14}C]dihydrokaempferol (Fig. 2) in the presence of 2-oxoglutarate, ascorbate and Fe^{2+} led to the formation of a new radioactive product. This product comigrated on TLC in the solvent systems 1–3 with the authentic flavonol kaempferol (Fig. 2) (for R_f values see ref. [4]). Furthermore, from [^{14}C]naringenin (Fig.2) as substrate the formation of dihydrokaempferol and kaempferol was observed, when enzyme preparations from lines with dominant alleles of both the *An3* and the *Fl* locus were used as enzyme source [15]. Highest enzyme activity for conversion of dihydrokaempferol to kaempferol was found with enzyme extracts from line V13 (Table III). Therefore, this line was used for the further characterisation of in vitro formation of flavonols.

The reaction was strictly dependent on the three cofactors 2-oxoglutarate, ascorbate and Fe^{2+}. Enzyme assays without these cofactors showed a considerably lower conversion of dihydrokaempferol to kaempferol (Table I). Moreover, when low molecular weight substances were removed by gel filtration on Sephadex G-50, no enzyme activity for flavonol formation could be observed. Addition of any of the cofactors alone did not restore enzyme activity. A small effect was observed after addition of a combination of 2-oxoglutarate and Fe^{2+}. Pronounced restoration of enzyme activity was only found in the presence of all three cofactors (Table I). In that case, kaempferol formation was even considerably higher than in the comparable assay with crude extract. This result is probably due to removal of substances which interfere with the enzymatic reaction.

With enzyme preparations from flowers of line V13 the synthesis of kaempferol was linear with protein concentration up to about 50 µg protein per assay and with time for about 10 min. Highest conversion of dihydrokaempferol to kaempferol was observed when the incubation was carried out at pH 6.5.

In inhibition experiments with dihydrokaempferol as substrate, strong inhibition was found with EDTA and diethyldithiocarbamate. Substantial inhibition

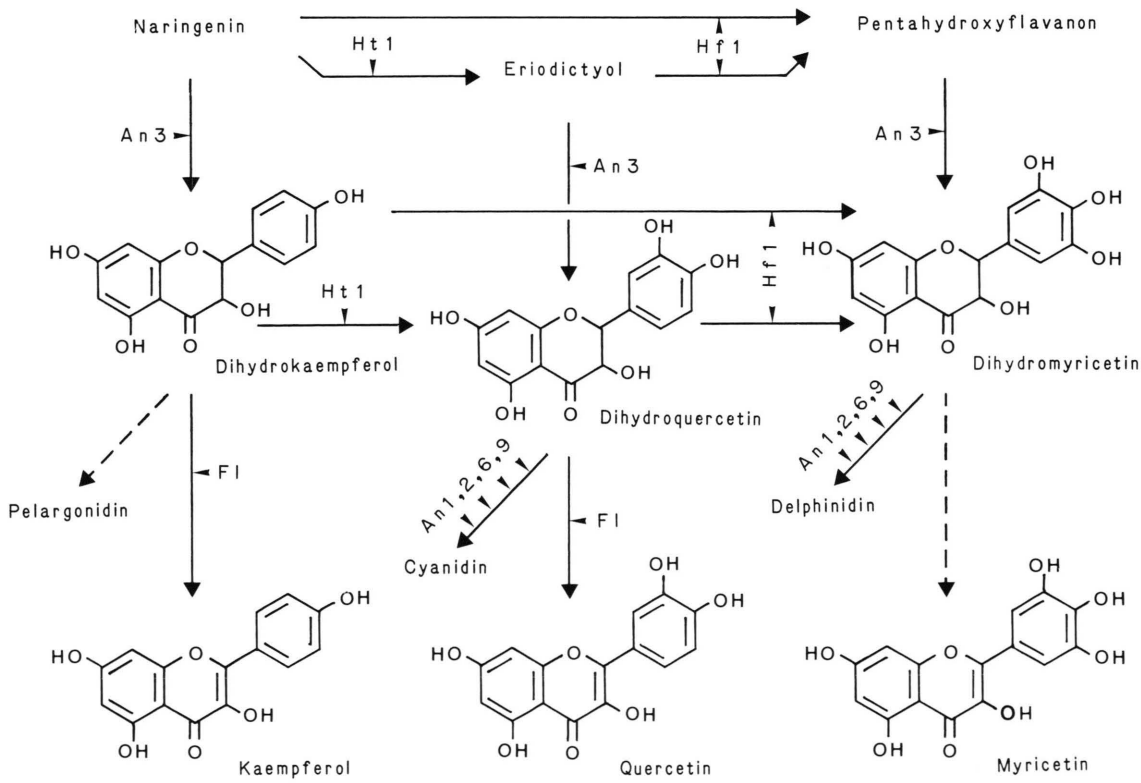

Fig. 2. Part of the biosynthetic pathway of flavonols and anthocyanins in *Petunia* and the localisation of some genes.

Table I. Cofactor requirement of enzyme activity for flavonol formation from flowers of line V 13.

Enzyme preparation	Cofactors added 2-Oxoglutarate	Ascorbate	Fe^{2+}	cpm in kaempferol[a]
Crude extract	+	+	+	1,472
	−	−	−	239
Crude extract after gel filtration (Sephadex G-50)	−	−	−	0
	+	−	−	0
	−	+	−	0
	−	−	+	0
	+	+	−	40
	+	−	+	620
	−	+	+	75
	+	+	+	3,127

[a] Reaction product formed with 40 µg protein after 5 min incubation.

was also observed upon addition of KCN or *p*-chloromercuribenzoate to the enzyme assays (Table II). These four substances also had inhibitory effect on the conversion of dihydrokaempferol to kaempferol in assays with enzyme preparations from parsley and from *Matthiola* [3, 4]. A clear stimulation was found in the presence of diethylpyrocarbonate (Table II). A similar effect of this compound has been reported for flavanone 3-hydroxylase activity from flowers of *Petunia* [15]. This enzyme also needs the three cofactors mentioned above.

Table II. Effect of various inhibitors on enzyme activity.

Additions	cpm in kaempferol	Relative activity [%]
None	2,463	100
2 mM KCN	1,679	68.2
5 mM KCN	1,017	41.3
0.5 mM EDTA	134	5.4
2 mM Diethyldithiocarbamate	136	5.5
0.1 mM *p*-Chloromercuribenzoate	1,163	47.2
0.5 mM Diethylpyrocarbonate	2,879	116.9

Flower extracts containing 10% (v/v) glycerol could be frozen in liquid nitrogen and stored for several weeks at −70 °C with about 35% loss of enzyme activity. Without glycerol a loss of about 50% was observed. When flowers were frozen in liquid nitrogen and stored at −70 °C, a loss of about 40% of extractable enzyme activity was found. The question was investigated whether dihydroquercetin and dihydromyricetin (Fig. 2) also serve as substrates for the conversion to the respective flavonols. Incubation of [^{14}C]dihydroquercetin with enzyme preparations from line V13 and the three cofactors led to the formation of quercetin (Fig. 2). Under standard conditions, the rate of conversion of dihydroquercetin to quercetin was similar to that of dihydrokaempferol to kaempferol. When, however, [^{14}C]dihydromyricetin was used as substrate, only a very small amount was found to be converted to the respective flavonol myricetin (about 13% in comparison to dihydrokaempferol or dihydroquercetin). Quercetin and myricetin formed in the enzyme assays were identified by cochromatography with authentic samples in the three solvent systems mentioned above.

The course of enzyme activity for flavonol formation as well as the flavonol content were studied during the development of buds and flowers. The developmental process was divided into eight morphologically different stages (Fig. 3) [15]. Flavonol is already present in the smallest buds (stage 1). Measured as A/g fresh weight, the amount of flavonol increases rapidly, reaches a maximum value at stage 4 and decreases continuously in the following stages (Fig. 3). However, the real amount of flavonol per flower is masked by the rapid increase of fresh weight in the last stages of flower development. Enzyme activity for flavonol formation increases rapidly from a low level in stage 1 to a clear maximum in stage 3. In enzyme preparations of the following stages, the enzyme activity decreases progressively to zero at stage 8 (Fig. 3). A similar course of enzyme activity was also found for flavanone 3-hydroxylase and flavonoid 3′-hydroxylase in flowers of *Petunia* [15, 14].

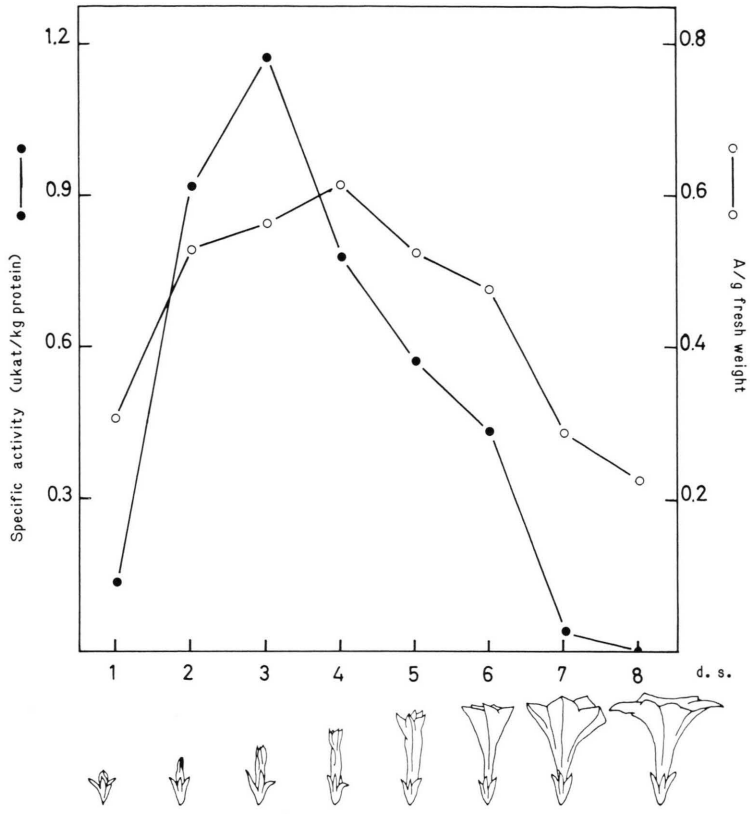

Fig. 3. The courses of flavonol content and enzyme activity for flavonol formation during bud and flower development in *Petunia*.
A = 372 nm; d.s. = developmental stages.

Incubations with enzyme preparations from other cyanic lines and also from acyanic lines with the dominant allele *Fl* led to essentially the same results as described for line V13 (Table III). Thus, the genes *An1* and *An2*, which block the anthocyanin pathway after dihydroflavonol formation (Fig. 2), do not interfere with the enzymatic conversion of dihydroflavonols to flavonols. In contrast, enzyme preparations from flowers of lines with recessive alleles (*fl/fl*) show under standard conditions only a very low enzyme activity for flavonol formation mostly not exceeding 10% of the activity found in line V13. With

Table III. Enzyme activity for conversion of dihydroflavonols to flavonols in flower extracts of different genotypes of *Petunia hybrida*.

Line code	Flower colour	Genotype[a] *Fl* locus	*An1*	*An2*	*An3*	*Ht1*	*Hf1*	Specific activity [µkat/kg protein]	Relative activity [%]
V13	purple bluish	*Fl/Fl*	+	+	+	+	+	1.39	100.0
V23	purple bluish	*Fl/Fl*	+	+	+	−	+	1.15	
V28	purple bluish	*Fl/Fl*	+	+	+	−	+	1.34	
W4	white	*Fl/Fl*	+	−	+	+	+	1.29	
W42	white	*Fl/Fl*	−	+	+	+	−	1.03	
V12	purple reddish	*fl/fl*	+	+	+	+	+	0.137	9.9
V33	purple purplish	*fl/fl*	+	+	+	+	+	0.089	6.4
R3	red	*fl/fl*	+	+	+	+	−	0.072	5.2
R4	very light red	*fl/fl*	+	+	+	−	−	0.289	20.8
R27	red	*fl/fl*	+	+	+	+	−	0.128	9.2
W39	white	*fl/fl*	+	+	−	+	−	0.059	4.3
W78	white	*fl/fl*	−	+	+	+	−	0.147	10.6

[a] + = homozygous dominant; − = homozygous recessive.

these enzyme preparations, clear conversion of dihydrokaempferol to kaempferol could only be achieved at longer incubation times (30 min to 1 h). Only line R4 (genotype *fl/fl*) showed also under standard conditions appreciable enzyme activity (Table III).

Discussion

Among the great number of genes known to control the biosynthesis of flavonoids in various plants a gene interfering with flavonol synthesis but not with anthocyanin formation is rather rare. Besides the gene *F* in *Cyclamen* [16, 17], the gene *Fl* in *Petunia* is now a chemogenetically well documented example. Flowers of genotypes with recessive alleles (*fl/fl*) still contain some flavonol, but the amount is greatly reduced in comparison to flowers with the dominant allele *Fl*. Thus, the synthesis of flavonols is obviously not completely blocked in the presence of recessive alleles.

Buds and flowers of *Petunia* already proved to be a valuable source for enzymes involved in flavonoid biosynthesis [18]. Now, we could also demonstrate enzyme activity for the conversion of dihydroflavonols to flavonols. As expected from the activity course of the respective enzyme in *Matthiola incana* [4] and from that of biosynthetically related enzymes in *Petunia* during bud and flower development [14, 15], enzyme activity could readily be demonstrated in extracts from small buds, but not in extracts from unfolded flowers. As found in parsley and in *Matthiola,* flavonol formation in *Petunia* is catalysed by a soluble enzyme which belongs according to its cofactor requirements to the 2-oxoglutarate dependent dioxygenases [19]. Furthermore, the pH at which maximal enzyme activity is exhibited is similar in the three plants and the reaction is strongly inhibited by the same compounds. From these results it can be concluded that in cell cultures of parsley and flower buds of *Matthiola* and *Petunia* the conversion of dihydroflavonols to flavonols is catalysed by the same type of enzyme. In contrast, a similar reaction, namely the oxidation of flavanones to flavones, is catalysed in parsley by a soluble dioxygenase [3], whereas in flowers of severeal plants an NADPH-dependent microsomal enzyme activity was shown to be responsible [20, 21].

Flavonols differ from dihydroflavonols only by a double bond between the carbon atoms 2 and 3. The fact that this reaction is catalysed by a dioxygenase is best rationalized by the assumption that the double bond of flavonols is introduced by hydroxylation in the 2-positioin with subsequent elimination of water. That means, two enzymes, a 2-hydroxylase and a dehydratase, should be involved in the formation of flavonols. Although the 2-hydroxylated dihydroflavonols expected as intermediates of the enzymatic reaction have been made chemically [22, 23], they were as yet not observed in respective enzyme assays and could also not be observed in our investigations on *Petunia*.

In agreement with the chemogenetic results described earlier and the genetic data presented in this paper, high enzyme activity for flavonol formation was only found in flower extracts prepared from genotypes with the dominant allele *Fl*. Moreover, enzyme preparations from recessive genotypes (*fl/fl*) were not devoid of this activity, but the *in vitro* conversion of dihydroflavonols to flavonols was drastically reduced. This results establish for the first time a correlation between a gene and enzymatic formation of flavonols. This correlation proves that the gene *Fl* of *Petunia* actually controls the step dihydroflavonol → flavonol and that the enzyme activity measured in the *in vitro* assay is definitely responsible for the oxidation of dihydroflavonols to flavonols *in vivo*.

The higher enzyme activity found in flower extracts of line R4 in comparison to the other lines with recessive *fl* alleles is in agreement with the observation that a moderate amount of kaempferol is present in the flowers of this line. But a high amount of dihydrokaempferol is also accumulated. This high dihydrokaempferol concentration might have a stimulating effect on enzyme synthesis or enzyme activity for flavonol formation.

Is has been concluded from chemogenetic investigations that the gene *Fl* controls the synthesis of kaempferol and quercetin but not the formation of myricetin [7]. Moreover, the latter flavonol was only present in minor amounts in the flowers. As expected from these results, dihydroquercetin, besides dihydrokaempferol, was found to be readily converted to the respective flavonol quercetin, whereas only a very low conversion rate of dihydromyricetin to myricetin was observed.

In flowers of genotypes dominant for both *Fl* and *Ht1* the amount of cyanidin was found to be greatly reduced in favour to quercetin [7]. This fact is clearly due to competition of the enzymes for flavonol for-

mation and for anthocyanin synthesis, for dihydroquercetin as common substrate (Fig. 2). For different reasons such a competition is not expected to occur for dihydrokaempferol and dihydromyricetin as substrates. Because pelargonidin is rarely found in *Petunia hybrida,* and if present, the amounts are very small [24], dihydrokaempferol is obviously not converted to the respective anthocyanidin but is an excellent substrate for kaempferol formation. In contrast, dihydromyricetin is readily converted to the respective anthocyanidin delphinidin, but was found to be a poor substrate for flavonol synthesis (Fig. 2). This relations confirm the observation that in flowers of *Petunia hybrida* derivatives of kaempferol, quercetin and delphinidin are the main endproducts of flavonoid biosynthesis.

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