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Genetic and biochemical studies on flavonoid 3′-hydroxylation in flowers of *Petunia hybrida*

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Summary. In flower extracts of defined genotypes of *Petunia hybrida,* an enzyme activity was demonstrated which catalyses the hydroxylation of naringenin and dihydrokaempferol in the 3′-position. Similar to the flavonoid 3′-hydroxylases of other plants, the enzyme activity was found to be localized in the microsomal fraction and the reaction required NADPH as cofactor. A strict correlation was found between 3′-hydroxylase activity and the gene *Ht1,* which is known to be involved in the hydroxylation of flavonoids in the 3′-position in *Petunia.* Thus, the introduction of the 3′-hydroxyl group is clearly achieved by hydroxylation of C_{15}-intermediates, and the concomitant occurrence of the 3′,4′-hydroxylated flavonoids quercetin and cyanidin (paeonidin) in the presence of the functional allele *Ht1* is due to the action of one specific hydroxylase catalysing the hydroxylation of common precursors for both flavonols and anthocyanins.

Key words: Anthocyanin biosynthesis – Flavonoid 3′-hydroxylase – Genetic control – *Petunia hybrida*

Introduction

In flowers of *Petunia hybrida,* a number of genes are known to modify the B-ring of flavonoids by hydroxylation and methylation. Of the three hydroxylation genes, *Ht1, Hf1* and *Hf2* mentioned by Wiering (1974), the gene *Ht1,* which is located on chromosome III (Maizonnier and Moessner 1979), has a phenotypical expression on flavonol, as well on anthocyanin level. Data from crossing experiments, however, have not been presented as yet. The data available so far suggest that mainly 4′-hydroxylated flavonoids are formed in recessive genotypes (*ht1ht1, hf1hf1, hf2hf2*). The dominant allele of the locus *Ht1* stimulates the synthesis of 3′,4′-hydroxylated compounds and in the presence of the dominant allele of the locus *Hf1* or *Hf2,* 3′,4′,5′-hydroxylated compounds are formed.

Hess (1968) concluded from studies with *Petunia* that the B-ring substitution pattern of flavonoids is already determined at the level of cinnamate derivatives. More recent results of complementation experiments and of chemogenetic investigations (Tabak et al. 1978; Doodeman et al. 1982) on white flowering mutants of *Petunia,* however, suggest that the appearence of the hydroxyl group at the 3′-position is most probably achieved after the synthesis of the flavonoid skeleton by a specific hydroxylation reaction on C_{15}-intermediates. Definite proof, however, can only be provided by enzymatic studies on defined genotypes of *Petunia.*

Although phenolases can catalyse hydroxylation of flavonoids in the 3′-position, they are obviously not specifically involved in flavonoid biosynthesis (Schill and Grisebach 1973). More recent studies on flowers of genetically defined plants, (Forkmann et al. 1980; Forkmann and Stotz 1981; Spribille and Forkmann 1982) and on cell suspension cultures (Hagmann et al. 1983; Fritsch and Grisebach 1975) prove that the introduction of the 3′-hydroxyl group is catalyzed by the enzyme flavonoid 3′-hydroxylase. In the plants so far investigated, flavonoid 3′-hydroxylase activity was found to be localized in the microsomal fraction and the reaction required NADPH as cofactor.

We now report on the further characterization of the genetic control of 3′-hydroxylation and on the demonstration of flavonoid 3′-hydroxylase activity in enzyme preparations from genetically defined flower colour mutants of *Petunia hybrida.*

Material and methods

Plant material

In Table 1 the genotypes and phenotypes of parents, F1's and other lines used are summarized.

Table 1. Genotype and phenotype of parents, F1's and other lines

Line code	Phenotype flower colour	HCC[a]	Anthocyanin		Flavonol[d]	Genotype[e]				
			Total[b]	Relative[c] amounts		*Ht1*	*Hf1*	*Hf2*	*Rt*	*An4*
R3	red 1	022/1	0.42	Cy 90 Dp 10	Qu	+	–	–	–	+
R4	very pale red	622/3	0.06	Cy 40 Dp 60	Km	–	–	–	–	+
R9	red 1	21/1	1.23	Cy 90 Dp 10	Qu	+	–	–	–	+
R27	red 1	20/1	0.47	Cy 90 Dp 10	Qu	+	–	–	–	–
M1	magenta	627	0.55	Pn 90 Mv 10	Qu	+	–	–	+	+
M7	very pale magenta	629/2	0.08	Pn 40 Pt 60	Km	–	–	–	+	+
V6	purple	732	5.4	Pt+Mv100	Qu	+	+	+	+	+
W37	white	–		–	–	–	–	–	–	+
W39	white	–		–	–	+	–	–	–	+
R4×R9	red 1	623	n.d.	Cy 90 Dp 10	Qu	±	–	–	–	+
M1×R4	magenta	26/1	n.d.	Pn 90 Mv 10	Qu	±	–	–	±	+
V6×R4	purple	732	n.d.	Pt+Mv 100	Qu	±	±	±	±	+

[a] HCC = Horticultural Colour Chart
[b] in optical density of 5.7 cm^2 flower limb in 10 ml methanol-HCl
[c] Cy = cyanidin, Dp = delphinidin, Pn = peonidin, Pt = petunidin, Mv = malvidin, in percentages of total
[d] Qu = quercetin, Km = kaempferol
[e] + = homozygous dominant, – = homozygous recessive, ± = heterozygous

Description of the gene action:

Ht1-: synthesis of quercetin and cyanidin or peonidin in flower limb and flower tube
ht1ht1: no or little synthesis of quercetin, accumulation of kaempferol, little synthesis of cyanidin and peonidin
Hf1-: synthesis of delphinidin, petunidin or malvidin in flower limb and flower tube
hf1hf1: no or little synthesis of delphinidin, petunidin or malvidin
Hf2-: synthesis of delphinidin, petunidin or malvidin in the flower limb, in smaller amounts than if *Hf1* is dominant
hf2hf2: no or little synthesis of delphinidin, petunidin or malvidin
Rt-: synthesis of anthocyanidin-3-rutinoside, which is a prerequisite for methylation of the positions 3′ and 5′
rtrt: anthocyanidin-3-glucoside present
An4-: anthocyanin present in flower tube and in the venation of the flower limb. If the dominant allele of the gene *Hf1* is present the pollen is coloured by anthocyanin
an4an4: less anthocyanin in the flower tube and in the venation of the limb, no anthocyanin in pollen

The action of genes in the biosynthetic pathway is shown in Fig. 1.

Chemicals and synthesis of substrates

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

(4a,6,8-^{14}C) Naringenin and (4a,6,8-^{14}C) dihydrokaempferol (both 3.09 GBq/mmol) were prepared enzymatically as described earlier (Forkmann et al. 1980, Britsch et al. 1981).

Preparation of the crude extract and the microsomal fraction

Five grams buds were homogenized in a prechilled mortar together with 2.5 g quartz sand, 2.5 g Dowex 1×2 and 25 ml 0.1 M potassium phosphate buffer, pH 7.5, containing 28 mM 2-mercaptoethanol. Centrifugation (20 min, 17,000×g) and filtration through glass wool provides the crude extract.

For the preparation of the microsomal fraction, 1 M $MgCl_2$ solution was added to the crude extract up to a final concentration of 30 mM and the mixture was gently shaked for 10 min. After centrifugation (20 min, 34,000×g) the pellet was suspended in 1/20 of the initial volume of the crude extract.

Assay for flavonoid 3′-hydroxylase

The assay system contained in 200 µl total volume: 20 µmol potassium phosphate buffer (pH 7.5), 0.28–1.4 µmol 2-mercaptoethanol, 0.06 nmol (10,000 cpm) radioactive substrate (naringenin or dihydrokaempferol, respectively), 10–30 µg protein and 0.1 µmol NADPH. Incubation was carried out for 5 min at 30 °C and was terminated by addition of 20 µl methanol containing a mixture of authentic flavonoids. The phenolics were extracted twice with ethyl acetate (100 µl + 50 µl) and chromatographed on cellulose plates in a solvent system chloroform/acetic acid/water (10:9:1). Radioactivity was localized by scanning the plates. The radioactive zones were scraped off and counted in Unisolve 1 in a scintillation counter.

Analytical methods

Anthocyanins and flavonols were analysed as described by Wiering and de Vlaming (1973) and Gerats et al. (1982). Protein was determinated according to Bradford (1976). For the identification of the reaction products radioactive zones were eluted with methanol and the products chromatographed

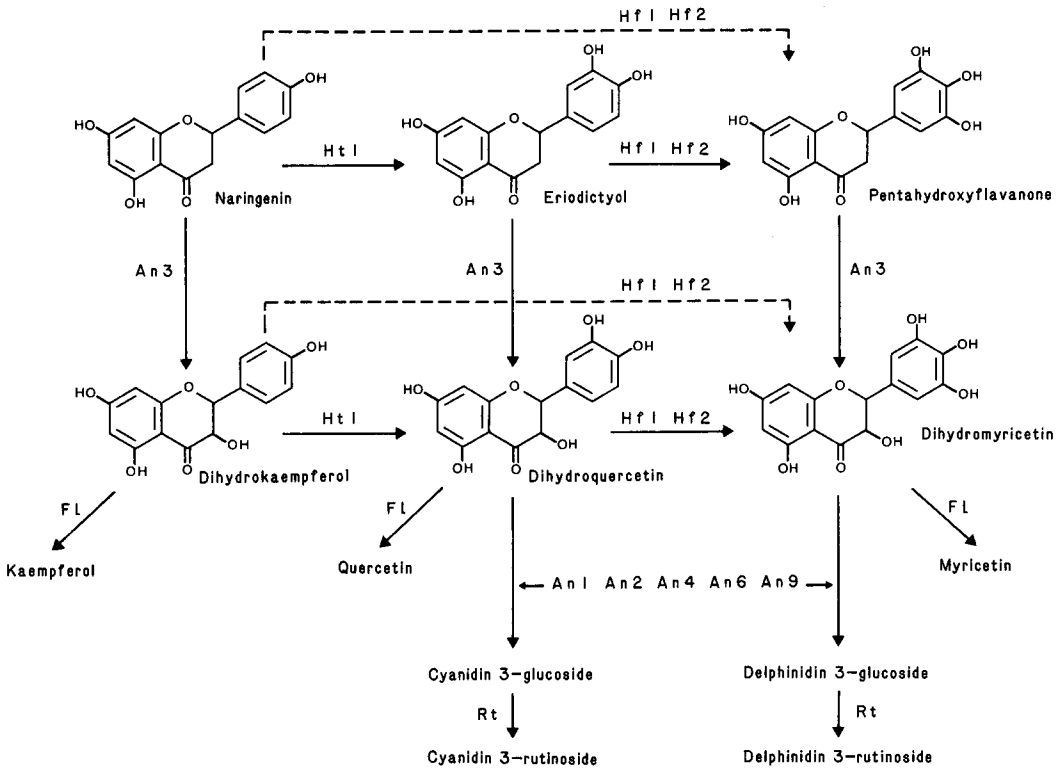

Fig. 1. Localisation of genes in the biosynthetic pathway of anthocyanins and flavonols in *Petunia hybrida*

with authentic samples in three different solvent systems (Forkmann et al. 1980). The products were also identified by enzymatic conversion to the respective flavone (Stotz and Forkmann 1981), dihydroflavonol (Forkmann and Stotz 1981) and flavonol (Spribille and Forkmann 1984).

Determination of the pH-optimum

The enzyme assays were carried out with 10 µl enzyme extract, 10 µl NADPH (dissolved in potassium phosphat buffer, pH 7.5) and 180 µl 0.1 M potassium phosphate buffer, pH 6.5–8.5.

Results

Genetic studies

In Table 2 the segregation for the gene *Ht1* in different crosses is given. In the F2 crosses (I) only *Ht1* segregates, in the B1 cross (II) *Ht1, Hf1* and *Hf2* segregate.

In the colour classes red 1 and magenta (*Ht1-, hf1hf1, hf2hf2*), dull red 2 and bluish magenta 1 (*Ht1-, hf1hf1, Hf2-*), a high amount of cyanidin derivatives (cyanidin and peonidin) occurs together with the flavonol quercetin. In these colour classes the dominant allele of *Ht1* is present. In the colour classes very pale red and very pale magenta (*ht1ht1, hf1hf1, hf2hf2*), dull red 3 and bluish magenta 2 (*ht1ht1, hf1hf1, Hf2-*), only small amounts of cyanidin derivatives are present together with the flavonol kaempferol; in these cases the recessive alleles of *Ht1* are present.

It should be noted that in the flowers of genotypes with recessive alleles of *Hf1* and *Hf2* some delphinidin derivatives are formed. This formation is independent on the genetic state of *Ht1*.

However, if the dominant allele of *Hf1* is present (the colour classes grey 1 and purple), the gene *Ht1* has no visible effect on the flower colour and only delphinidin derivatives (delphinidin, petunidin and malvidin) are present. The nature of the flavonol, however, is still dependent on the genotype with regard to the gene *Ht1* (Table 2).

There is a monofactorial segregation for the gene *Ht1* in the F2's and B1 (see also Wiering and de Vlaming 1977). Moreover, the genes *Ht1, Hf1* and *Hf2* are independent (Table 2).

Biochemical studies

Preliminary investigations on flower extracts of commercial strains of *Petunia* revealed the presence of two enzyme activities which catalyse hydroxylation of flavonoids in the 3′ and the 3;5′-position, respectively (Stotz, unpublished). From the genetic data, it can be assumed that these hydroxylating enzymes are controlled by the genes *Ht1* and *Hf1*, respectively. By the use of suitable genotypes it should be possible to separate the two enzyme activities. Therefore, the enzyme extracts for the determination of flavonoid 3′-hydroxylase activity were prepared from flowers of genotypes with the

Table 2. The F2 composition of crosses (R4 × R9) and (M1 × R4) designated I and of backcross (V6 × R4) × M7 designated II

Expected segregating types					Results No. of plants	
Genotype	Phenotype flower colour		Relative amounts of anthocyanins[a]	Main flavonol[b]	Cross I	Cross II
ht1ht1hf1hf1hf2hf2	very pale red	+ very pale magenta	C 40 D 60	Km	59	20
ht1ht1hf1hf1Hf2–	dull red 3	+ bluish magenta 2	C 10 D 90	Km	–	24
ht1ht1Hf1– – –	grey 1	+ purple	C 0 D 100	Km	–	40
Ht1 – hf1hf1hf2hf2	red 1	+ magenta	C 90 D 10	Qu	153	28
Ht1 – hf1hf1Hf2–	dull red 2	+ bluish magenta 1	C 40 D 60	Qu	–	37
Ht1 – Hf1 – – –	grey 1	+ purple	C 0 D 100	Qu	–	43
				Total	212	192

Segregation for *Ht1* Crosses I 153: 59 $P^{d}_{3:1} = 0.34$
Cross II 108: 84 $P_{1:1} = 0.08$
Hf1 Cross II 83:109 $P_{1:1} = 0.07$
Hf2[c] Cross II 61: 48 $P_{1:1} = 0.22$

Linkage *Ht1-Hf1* 43:65:40:44 $P_{2\times2} = 0.19$
Ht1-Hf2 37:28:24:20 $P_{2\times2} = 0.80$

[a] C = cyanidin derivatives, D = delphinidin derivatives, in percentages of total
[b] Km = kaempferol, Qu = quercetin
[c] in *hf1hf1* only
[d] the p values are at the χ^2 level

Table 3. Cofactor requirement and subcellular localisation of the 3′-hydroxylase activity

Enzyme source	Cofactor added	3′-Hydroxylase activity (μkat/kg protein)
Crude extract	none	0
	NADPH	1.24
Supernatant of microsomal pellet	NADPH	0.5
Microsomal pellet	none	0
	NADPH	5.3
	NADH	0.58

dominant allele *Ht1* but recessive alleles of the gene *Hf1* and *Hf2* (lines R3, R27 and W39; Table 1).

When a crude extract from line R3 was incubated with (^{14}C) naringenin in the presence of NADPH the radiochromatogram of the reaction mixture showed a new compound. This compound was identified as eriodictyol by co-chromatography with the authentic flavanone in 3 different solvent systems and by enzymatic conversion to luteolin and dihydroquercetin (Forkmann et al. 1980; Stotz and Forkmann 1981).

After preparation of the microsomal fraction by Mg^{2+}-precipitation the 3′-hydroxylase activity was present in the microsomal pellet (Table 3, Fig. 2a). In incubations with the microsomal fraction, a variable but always small amount of a radioactive product was found to be formed in addition to eriodictyol (Fig. 2a). On co-chromatography this product did not separate from authentic 5,7,3′,4′,5′-pentahydroxyflavanone. Hydroxylation on 3′-position was strictly dependent on NADPH. Substitution of NADPH by NADH strongly reduced the formation of eriodictyol (Table 3).

Under standard conditions the reaction was linear with protein concentration up to about 20 μg microsomal protein per assay. Strong linearity with time was not observed. This non-linearity seems to be mainly due to the temperature sensitivity of the microsomal preparation. Thus, after preincubation of the microsomal fraction at 30 °C for 10 min, 40–55% of the originally present enzyme activity was lost. Similar results have been reported for the flavonoid 3′-hydroxylase from other plants (Forkmann et al. 1980; Stotz 1983). With naringenin as substrate and at different incubation times not exceeding 12 min, maximal activity was exhibited at pH values around 7.5. The reaction was partially inhibited by diethylpyrocarbonate. In addition to naringenin, dihydrokaempferol was found to be a substrate for 3′-hydroxylation. The reaction product formed was identified as dihydroquercetin by co-chromatography in three different solvent systems as mentioned above and by enzymatic conversion to the respective flavonol quercetin (Spribille and Forkmann 1984).

One of the interesting features of 3′-hydroxylase to elucidate is the substrate specificity of the enzyme. Data on the activities with different substrates could answer the question whether flavanones or dihydroflavonols act as in vivo substrates for 3′-hydroxylation. From three experiments using naringenin and dihydrokaempferol as substrates mean K_m values of respectively 0.39×10^{-6} M ± 0.08 and 1.68×10^{-6} M ± 0,87 and mean V values of respectively 0.35 pkat ± 0.08 and 0.31 pkat ± 0.14 were found.

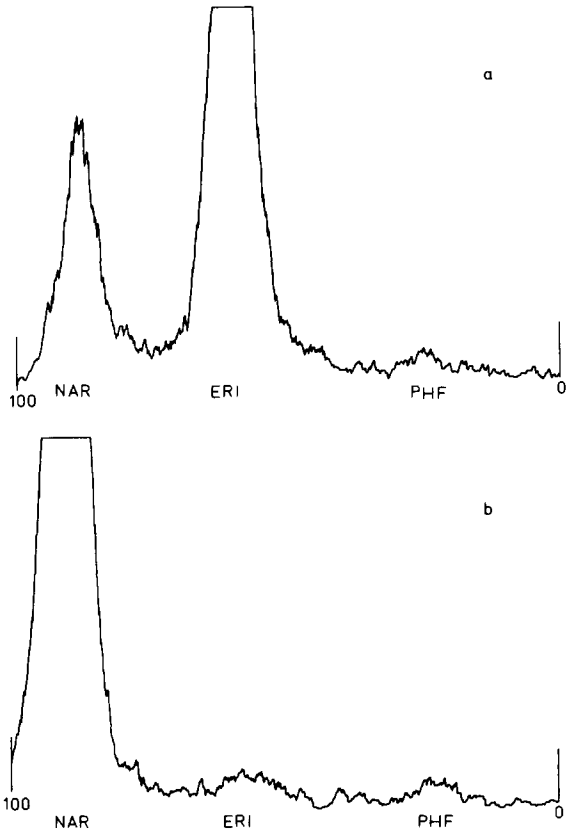

Fig. 2a, b. Radioscan of TLC on cellulose with solvent system chloroform/acetic acid/water, (10:9:1) from incubation of (^{14}C) naringenin with microsomal fraction from flowers of *P. hybrida* in the presence of NADPH. **a** line R3 (*Ht1*), **b** line R4 (*ht1ht1*); NAR: naringenin, ERI: eriodictyol; PHF: 5,7,3′,4′,5′-pentahydroxyflavanone

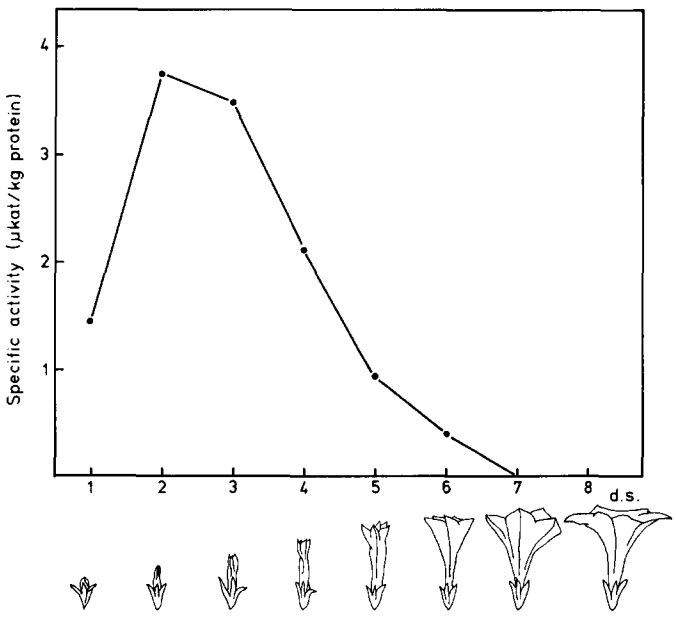

Fig. 3. The course of flavonoid 3′-hydroxylase activity during bud and flower development. (d.s.: developmental stages)

The enzyme activity for 3′-hydroxylation was measured during the development of buds and flowers. Morphological criteria were used to divide the organ development into eight significantly different stages (Fig. 2). Flavonoid 3′-hydroxylase activity is already present in the buds of stage 1 and increases rapidly to a maximum at stages 2 and 3. In enzyme preparations of the following stages, the enzyme activity decreased rapidly to zero at stages 7 and 8 (Fig. 3). A similar course of enzyme activity was also found for flavanone 3-hydroxylase (Froemel et al., in preparation) an enzyme which acts like flavonoid 3′-hydroxylase on flavanones in the biosynthetic pathway, whereas enzymes catalysing the terminal steps of anthocyanin biosynthesis were found to exhibit highest activity at later stages of bud development (Gerats et al. 1983).

Incubations with crude extracts or microsomal fractions prepared from flowers of line R27 and W39 led to essentially the same results as described for line R3. Thus, the genes *An4* and *An3*, respectively, do not interfere with 3′-hydroxylation. However, when the incubations were carried out with enzyme preparations from the lines R4 and W37 with recessive alleles of *Ht1*, only a very low formation of eriodictyol was observed beside the small amount of pentahydroxyflavanone mentioned above (Fig. 2 b). In the meantime it could be proved that the small amount of eriodictyol is not formed by action of 3′-hydroxylase but is as well as pentahydroxyflavanone due to a residual flavonoid 3′,5′-hydroxylation activity in the presence of recessive alleles of *Hf1* (Stotz et al., in preparation).

Discussion

The genetic data presented in this paper provide evidence for a concomitant occurrence of the 3′,4′-hydroxylated flavonoids quercetin and cyanidin (paeonidin) in the presence of the dominant allele *Ht1*. But in the presence of recessive alleles only the 4′-hydroxylated flavonol kaempferol is formed in addition to small amounts of cyanidin (paeonidin). In this context, it should be noted that the 4′-hydroxylated anthocyanidin pelargonidin is rarely found in *Petunia hybrida*, and, if present, the amounts are very small (Cornu *et al.* 1974). From the genetic data, however, we cannot exclude that two closely linked genes are involved in 3′-hydroxylation in *Petunia*, one gene responsible for flavonols and the other for anthocyanins.

From the biochemical results, it can be assumed that in both *Petunia* and the plants investigated earlier, the hydroxylation of naringenin and dihydrokaempferol in 3′-position is catalysed by the same type of enzyme, which is most probably a cytochrome P-450-dependent monooxygenase (Hagmann et al. 1983).

It is an interesting feature that no significant differences in kinetic parameters were observed between naringenin and dihydrokaempferol when used as substrates for 3′-hydroxylation. This particular result is not indicative for a preferential stage of 3′-hydroxylation during biosynthesis of flavonoids. The observation, however, that higher B-ring hydroxylated flavanones are poor substrates for 3-hydroxylation (Froemel et al., in preparation) might suggest a 3′-hydroxylation at a later stage than flavanones.

The occurrence of mainly 4′-hydroxylated flavonoids in the flowers of the triple recessive genotype (*ht1ht1, hf1hf1, hf2,hf2*) is clearly due to the complete lack of flavonoid 3′-hydroxylase activity. The small amounts of flavonoids with higher B-ring hydroxylation pattern in these flowers (Tables 1 and 2) are formed by action of flavonoid 3′,5′-hydroxylase which is not completely inactive in the presence of recessive *Hf1* alleles (Stotz et al., in preparation). In contrast to the lines with recessive alleles of the hydroxylation genes, however, flavonoid 3′-hydroxylase activity could be demonstrated in all investigated lines with the dominant allele *Ht1*. Furthermore, as predicted from earlier chemogenetic studies and complementation experiments (Tabak et al. 1978, Doodemann et al. 1982) on *Petunia*, naringenin and dihydrokaempferol were found to be substrates for the enzymatic hydroxylation in 3′-position. These results prove not only that the enzyme activity measured in the in vitro assay is definitely responsible for 3′-hydroxylation in vivo but also that in *Petunia* the introduction of the 3′-hydroxyl group is clearly achieved by hydroxylation of C_{15}-intermediates. These intermediates are common precursors for both anthocyanins and flavonols. Therefore, the concomitant occurence of quercetin and cyanidin is due to the action of one specific hydroxylase which is controlled by the gene *Ht1*.

With regard to 3′-hydroxylation, similar results were found at enzymatic investigations on defined genotypes of *Matthiola incana* (Forkmann et al. 1980), *Antirrhinum majus* (Forkmann and Stotz 1981) and *Dianthus caryophyllus* (Spribille and Forkmann 1982). In contrast, genetic and biochemical studies on flowers of *Silene dioica* indicate that in this plant the hydroxylation of flavonoids in 3′-position is determined at the 4-coumaroyl-CoA stage (Kamsteeg et al. 1980).

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