Flavonol Synthase Activity and the Regulation of Flavonol and Anthocyanin Biosynthesis during Flower Development in *Dianthus caryophyllus* L. (Carnation)

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Flavonol synthase (FLS) was demonstrated in crude extracts from flower buds of *Dianthus caryophyllus* (carnation). The enzyme catalyzed the conversion of dihydrokaempferol and dihydroquercetin to kaempferol and quercetin, respectively. The reaction required 2-oxoglutarate, ferrous ion and ascorbate as co-factors and had a pH optimum at about 7.4. The demonstration of FLS activity allowed comparative studies on flavonol and anthocyanin biosynthesis during bud and flower development. Besides FLS the flavonoid enzymes chalcone synthase (CHS), flavanone 3-hydroxylase (FHT) and dihydroflavonol 4-reductase (DFR) were measured. DFR is specifically involved in anthocyanin synthesis, while CHS and FHT provide dihydroflavonol, the common substrate for both FLS and DFR. Maximum expression of CHS, FHT and FLS activity was already observed in small buds, whereas DFR activity started to increase much later and reached its highest level in opened flowers. A substantial correlation was observed between the time courses of FLS and DFR activity and the accumulation of flavonols and anthocyanins, respectively. The competition of FLS and DFR for dihydroflavonols was found to be largely circumvented by different substrate specificities and by the sequential expression of the two enzymes. Both flavonols and anthocyanins are obviously not, or only to some extent, subject to degradation.

Introduction

During the last twenty years considerable progress has been made in elucidating enzymology and regulation of flavonoid biosynthesis. Initially, flavonoid enzymes were mainly demonstrated in protein preparations from illuminated cell suspension cultures [1]. But more recent work revealed that flowers are also rich sources of flavonoid enzymes [2]. This now allows successful enzymological studies on genetically defined material, and the extensive genetic information available has thus been turned to advantage in linking the presence or absence of particular enzyme activities with known genetic blocks along the pathway.

Dianthus caryophyllus (carnation) belongs to those plant species, in which flavonoid biosynthesis has been well investigated. The end products of flavonoid metabolism in carnation flowers are glycosides of the anthocyanidins pelargonidin and cyanidin as well as of the flavonols kaempferol and quercetin (Fig. 1). By chemogenetic studies on differently coloured varieties, Geissman and Mehlquist [3, 4] have identified a number of genes controlling biosynthesis and modification of flavonoids in carnation. Besides other genes, they reported on three complementary acting basic genes (Y, I and A) for anthocyanin formation. The function of gene Y is still unclear. It is obvious, however, that it influences flavonoid concentration. The genes I and A are structural genes of chalcone isomerase (CHI) and dihydroflavonol 4-reductase (DFR), respectively [5, 6]. CHI catalyzes the cyclization of chalcones to flavanones, and DFR reduces dihydroflavonols to flavan-3,4-diols (Fig. 1). In addition to CHI and DFR, the key enzyme of flavonoid biosynthesis, chalcone synthase (CHS), and the flavonoid modifying enzyme, flavonoid 3′-hydroxylase (F3′H), have been

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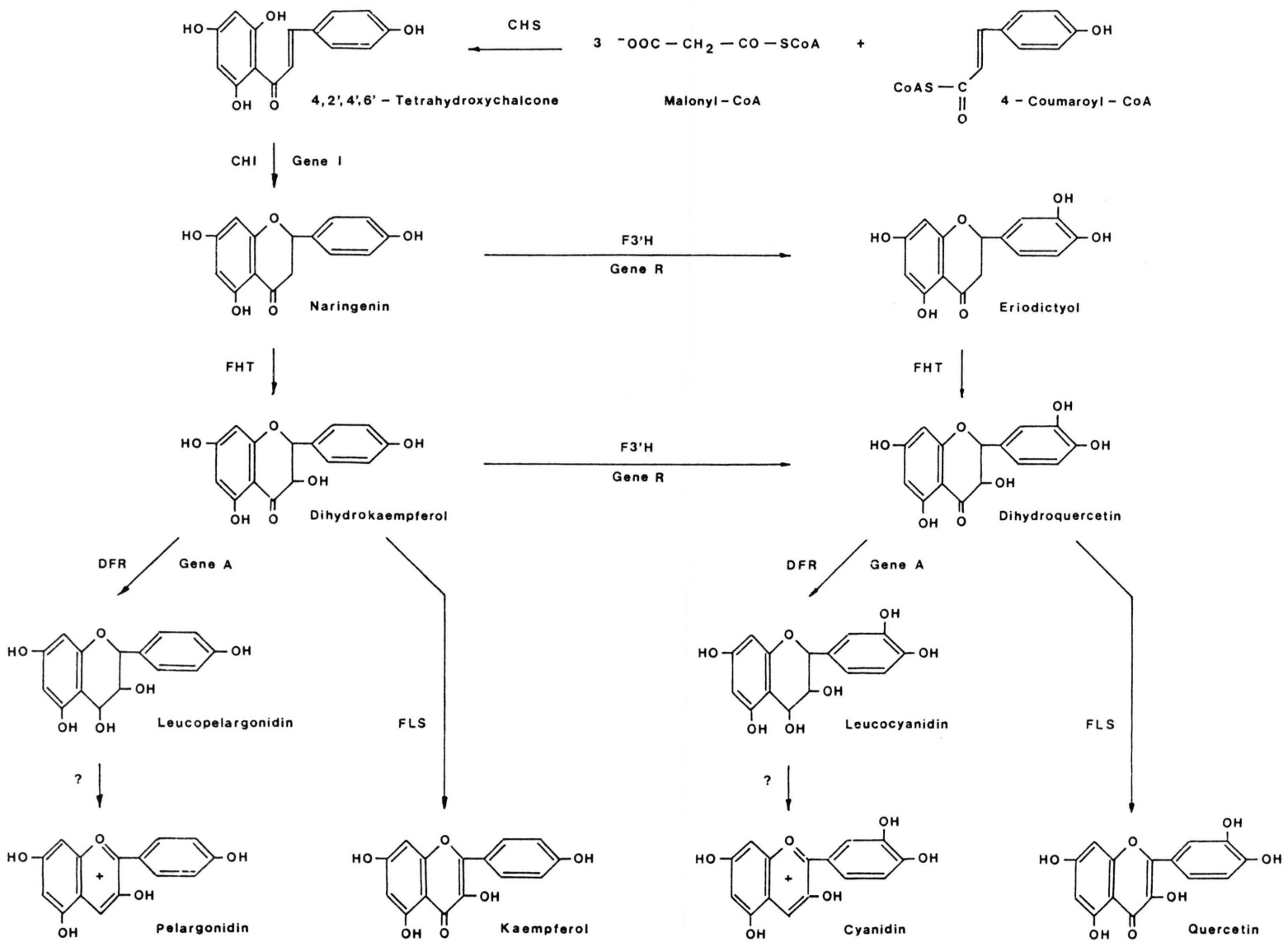


Fig. 1. The biosynthetic pathway to the flavonoid end products kaempferol and quercetin (flavonols) as well as pelargonidin and cyanidin (anthocyanidins), the enzymes involved in their biosynthesis and the genes controlling specific steps in flowers of carnation. CHS: chalcone synthase; CHI: chalcone isomerase; FHT: flavanone 3-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol 4-reductase; F 3'H: flavonoid 3'-hydroxylase; ?: unknown enzyme(s).

demonstrated in extracts of carnation flowers [7], and very recently, flavanone 3-hydroxylase (FHT), catalyzing the conversion of flavanones to dihydroflavonols, could also be characterized [8]. Thus all known enzymes of anthocyanidin biosynthesis have been studied in flowers of carnations. However, the key enzyme in flavonol formation, flavonol synthase (FLS), has not yet been investigated (Fig. 1). Until now FLS activity has been shown to be present in cell suspension cultures of parsley [9] and in flower extracts of *Matthiola incana* [10] and *Petunia hybrida* [11].

We have now been able to demonstrate FLS activity in flower buds of carnation but not in open flowers, in which DFR is active [6]. In order to get a more detailed insight in the relationship between enzyme activity and flavonoid accumulation during bud and flower development, we characterized the time course of FLS activity along with the activities of CHS including CHI, FHT and DFR as well as the accumulation of flavonols and anthocyanins. DFR and FLS are specifically involved in anthocyanin and flavonol formation, respectively, whereas CHS and FHT provide dihydroflavonol, the common precursor for both end products. The expected competition of FLS and DFR for dihydroflavonols as substrates was found to be largely circumvented by sequential induction and different substrate specificities of the two enzymes. The knowledge of the time course of enzyme activity and flavonoid accumulation is critical for our efforts to demonstrate still unknown flavonoid enzymes, to isolate flavonoid specific cDNA clones and to study gene action and regulation at the molecular level.

Materials and Methods

Plant material

The investigations were performed with the commercial variety "Tanga" (Barbaret & Blanc, Antibes, France), which contains pelargonidin and kaempferol derivatives as flavonoid end products in the flowers. The plant material was cultivated in a greenhouse.

Stages of flower development

Since the velocity of bud and flower development of carnation depends on environmental factors, morphological criteria were used for subdividing the developmental process into 9 significantly different stages.

Stage 1: closed buds with 5 mm diameter.
Stage 2: closed buds with 7 mm diameter.
Stage 3: closed buds with 10 mm diameter.
Stage 4: closed buds shortly before opening. The petals in the buds are still white.
Stage 5: just opening buds. The petals in the buds are pink.
Stage 6: opening buds. The visible part of the petals is 1 mm long.
Stage 7: the visible part of the petals is 5 mm long.
Stage 8: the visible part of the petals is 10 mm long.
Stage 9: the visible part of the petals is 15 mm long.

Chemicals and synthesis of substrates

Naringenin, dihydroquercetin, kaempferol and quercetin were obtained from Roth (Karlsruhe, F.R.G.). Dihydrokaempferol, leucopelargonidin and 4-coumaroyl-CoA were from our laboratory collection.

Labeled naringenin, dihydrokaempferol and dihydroquercetin were prepared enzymatically from [^{14}C]malonyl-CoA (2.22 GBq/mmol; Amersham-Buchler, Braunschweig, F.R.G.) and 4-coumaroyl-CoA as described [12–15].

Characterization of flavonol synthase (FLS)

The preparation of crude enzyme extracts and the enzyme assays were performed according to [10, 11], with the exception that 0.1 M potassium phosphate buffer, pH 7.4, was used. The dependence of the reaction on pH was determined in mixtures of 165 µl of 0.1 M potassium phosphate buffer (pH between 6.0 and 8.6), 30 µl aqueous co-factor solution and 5 µl crude extract.

Flavonoid enzyme activities during bud and flower development

In order to ensure comparable conditions, buds and flowers of the 9 developmental stages were collected at the same time. Each stage was subdivided in 5 samples. Four of them were immediately processed for the demonstration of the 4 different flavonoid enzymes investigated. The remaining sample was used for the determination of the anthocyanin and flavonol content. Standard

procedures were used for the preparation of enzyme extracts and the enzyme assays of CHS [7], FHT [8], DFR [6] and FLS (see above). The assay conditions ensured linearity with incubation time and protein concentration. All tests were run in duplicate. The measurements were confirmed by a second independent preparation of enzyme extracts.

Analytical methods

The anthocyanin and flavonol content of the petals was determined spectrophotometrically as described by Forkmann and Seyffert [16].

TLC of substrates and products was performed on precoated cellulose plates (Merck, Darmstadt, F.R.G.). The following solvent systems were used: (1) chloroform/acetic acid/water (10:9:1); (2) 30% acetic acid; (3) acetic acid/conc. HCl/water (30:3:10); (4) *n*-butanol/acetic acid/water (4:1:5, upper phase); (5) *tert*-butanol/acetic acid/water (3:1:1).

Radioactivity was localized by scanning the plates (TLC Analyzer, Berthold, Wildbad, F.R.G.) and enzyme activity was determined by integration of the peak areas of the substrates and products. Protein was determined by a modified Lowry procedure [17].

Results

Demonstration of flavonol synthase

Dihydroflavonols are the immediate precursors for flavonol formation as well as intermediates in anthocyanin synthesis. They are provided by the reaction sequence shown in Fig. 1 which includes the enzymes CHS, CHI and FHT.

Investigations on the course of FHT activity during bud and flower development of carnation revealed the presence of an as yet not detected enzyme activity. When enzyme preparations from flowers were incubated with [^{14}C]naringenin in the presence of the co-factors of the FHT reaction, 2-oxoglutarate, ferrous ion and ascorbate, the expected formation of dihydrokaempferol was observed. When, however, small buds were used as enzyme source, besides dihydrokaempferol a second radioactive product was detected, which was identified as the flavonol kaempferol by co-chromatography with an authentic sample in five different solvent systems (Table I). Thus, kaempferol is obviously formed from naringenin *via* dihydrokaempferol by the combined action of FHT and FLS. In confirmation of this result, [^{14}C]dihydrokaempferol was readily converted to kaempferol in the presence of the co-factors mentioned above by enzyme preparations from small buds but not from open flowers. Therefore, enzyme preparations from stage 2–4 were used for the further characterization of *in vitro* formation of the flavonol.

Table I. R_f-values (× 100) on cellulose plates of substrates and products.

Compound	Solvent system[a]				
	1	2	3	4	5
Dihydrokaempferol	76	68	85	88	86
Dihydroquercetin	43	65	81	84	79
Kaempferol	66	12	65	80	79
Quercetin	22	7	45	74	63

[a] See Materials and Methods.

The reaction was strictly dependent on the three co-factors 2-oxoglutarate, ferrous ion and ascorbate (Table II). In enzyme assays without these co-factors, only very low rates of flavonol formation could be observed. No FLS activity was detectable, when low molecular weight substances were removed by gel filtration (Sephadex G-50). Addition of only one of the co-factors did not restore enzyme activity. A considerable conversion of dihydrokaempferol to kaempferol occurred, when a combination of 2-oxoglutarate and ascorbate was added to the enzyme assay. But full restoration of enzyme activity could only be obtained by addition of all three co-factors (Table II).

For the conversion of dihydrokaempferol to kaempferol a broad pH optimum curve with a maximum at pH 7.4 was found. Linearity of the reaction with protein concentration and incubation time was observed for up to 50 µg protein per assay and a period of 15 min. Strong inhibition of the reaction was found with EDTA, diethyldithiocarbamate, *p*-hydroxymercuribenzoate, 2,2′-dipyridil, and KCN inhibited substantially (Table III). No decrease of enzyme activity was detected, when the petals were frozen in liquid nitrogen and stored at −70 °C for several weeks. In genotypes with the dominant allele of the gene R, kaempferol and quercetin derivatives are present in the petals. This is due to the flavonoid modifying enzyme F 3′H ca-

Table II. Co-factor requirement of the enzyme activity for kaempferol formation from dihydrokaempferol in extracts from small buds (stage 2–4).

Enzyme source	Co-factor added[b]	Enzyme activity (Bq in kaempferol formed from 167 Bq dihydrokaempferol)[a]
Crude extract	none	1.2
	ascorbate and 2-oxoglutarate and Fe^{2+}	31.6
Crude extract after gelfiltration (Sephadex G 50)	none	0
	ascorbate	1.8
	2-oxoglutarate	1.6
	Fe^{2+}	1.5
	ascorbate and 2-oxoglutarate	18.4
	ascorbate and Fe^{2+}	1.0
	2-oxoglutarate and Fe^{2+}	2.1
	ascorbate and 2-oxoglutarate and Fe^{2+}	31.0

[a] Product formed in 15 min with 40 µg protein.
[b] The enzyme system contained in 200 µl total volume 0.2 nmol [^{14}C]dihydrokaempferol, 50 nmol 2-oxoglutarate, 10 nmol $FeSO_4$ and 1 µmol ascorbate.

Table III. Effect of various inhibitors on FLS activity.

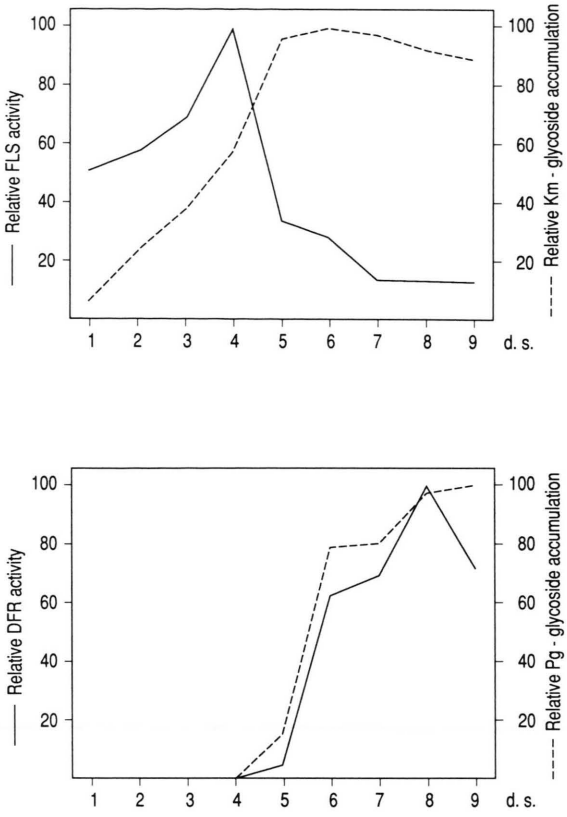
Additions	Enzyme activity [%][a]
None	100
5 mM KCN	27
0.5 mM EDTA	6
2 mM DDC	20
2 mM DPC	84
0.1 mM *p*-OHMB	11
2 mM 2,2′-dipyridil	10

[a] 100% = 0.6 µkat/kg protein.
DDC, diethyldithiocarbamate; DPC, diethylpyrocarbonate; *p*-OHMB, *p*-hydroxymercuribenzoate.

talyzing the hydroxylation of dihydrokaempferol to dihydroquercetin [7]. Therefore, it was investigated whether dihydroquercetin serves as substrate for quercetin formation. Incubation of [^{14}C]dihydroquercetin with bud extracts and the three co-factors led to the formation of quercetin, which was identified by co-chromatography with an authentic sample in five different solvent systems (Table I). Under standard conditions, the conversion rates of dihydrokaempferol to kaempferol (0.60 µkat/kg protein), and of dihydroquercetin to quercetin (0.63 µkat/kg protein) were essentially the same.

Time course of flavonoid accumulation and enzyme activity

Flavonol and anthocyanin accumulation as well as the changes in the activities of the enzymes involved in their biosynthesis were studied during bud and flower development of the variety "Tanga" (Fig. 2). This variety possesses the recessive alleles (rr) of the gene R and therefore accumulates only kaempferol and pelargonidin derivatives as flavonoid end products in the flowers. Subdivision of the developmental process into 9 morphologically defined stages gave a good agreement of independent measurements and led to characteristic activity curves. The flavonol and anthocyanin content were related to the fresh weight of the petals at each stage. Since the protein content of the enzyme extracts decreased rapidly during development, the enzyme activities were not related to protein, but rather calculated on fresh weight basis as well.



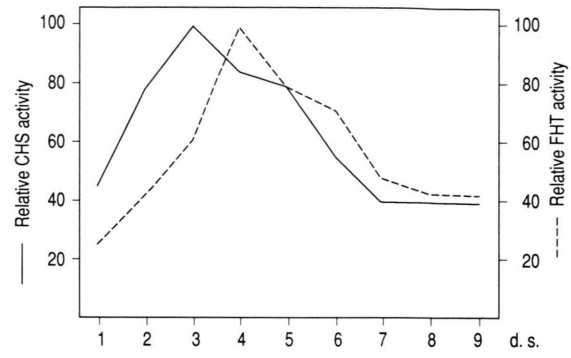


Fig. 2. The course of flavonoid enzyme activities and flavonoid accumulation in 9 developmental stages of buds and flowers of carnation. The enzyme activities are calculated on a fresh weight basis. Flavonols and anthocyanins were measured at $A_{364\,nm}$ and $A_{510\,nm}$, respectively. All values are given in percent of the maximum values attained.
CHS 100% = 0.8 μkat/kg
FHT 100% = 8.8 μkat/kg
DFR 100% = 0.2 μkat/kg
FLS 100% = 1.1 μkat/kg
d. s.: developmental stage;
a: relative FLS activity and flavonol accumulation (Km = kaempferol);
b: relative DFR activity and anthocyanin accumulation (Pg = pelargonidin);
c: relative CHS and FHT activities.

Even the smallest buds (stage 1) were found to contain kaempferol derivatives. In the following stages the flavonol concentration increased rapidly and reached a maximum at stage 6 and remained at a high level in the later stages (Fig. 2a). FLS, which is immediately responsible for flavonol formation, showed high activity already at stage 1. In the next stages FLS activity first increased slowly and then rapidly to reach its maximum at stage 4 (1.1 μkat/kg protein with dihydrokaempferol as substrate). In the further course of flower development, a fast decrease occurred with a drastic reduction of about 70% of the enzyme activity between stages 4 and 5. In opening flowers, FLS activity remained constant, but at a very low level (Fig. 2a).

In contrast to flavonol formation, the synthesis of anthocyanins (pelargonidin derivatives) started relatively late during bud and flower development. Anthocyanins could first be observed in buds of stage 5. The amount of anthocyanin, being rather low in stage 5, already reached nearly 80% of its maximal value at stage 6. This drastic increase was followed by a smaller increase in the anthocyanin content till stage 9 (Fig. 2b). DFR, the key enzyme of the anthocyanin pathway, exhibited a course of activity during the developmental process, which is nearly identical with that of anthocyanin accumulation. Thus only a weak DFR activity was found at stage 5. In enzyme extracts of the following stages the enzyme activity increased rapidly and the maximal activity (0.2 μkat/kg protein with dihydrokaempferol as substrate) was observed at stage 8 followed by a pronounced reduction in stage 9 (Fig. 2b).

Since dihydroflavonols may be converted to either flavonols or anthocyanins, they represent the branch point in the formation of the two end products of flavonoid biosynthesis in carnation. Therefore, the course of enzyme activities involved in the synthesis of these important intermediates is of particular interest. The enzymes responsible for dihydroflavonol formation are CHS, CHI and FHT (see Fig. 1).

Because not chalcone but flavanone formation was measured, the CHS-reaction also included CHI. An appreciable enzyme activity was already found in petal extracts from buds of stage 1. From this relatively high level, CHS activity increased rapidly to a distinct maximum at stage 3 (0.8 μkat/kg protein). In enzyme preparations of the following stages CHS activity decreased slowly and it remained constant on a considerable level up to stage 9 (Fig. 2c). The course of FHT activity during bud and flower development is similar to that of CHS, except that the activity in stage 1 is lower and that the maximum is only reached at stage 4 (8.0 μkat/kg protein). Despite the significant decline of FHT activity in the following three stages, enzyme activity remained relatively high till stage 9 (Fig. 2c).

Discussion

The major flavonoid compounds in carnation flowers are flavonols and anthocyanins. For the demonstration of enzymes involved in anthocyanin biosynthesis, flower extracts have proved to be an excellent source [2]. Now, we could also demonstrate FLS activity catalyzing the conversion of dihydroflavonols to flavonols. In contrast to anthocyanin formation, however, this enzyme activity was only detected in extracts from small buds. In *Matthiola* and *Petunia,* FLS expression was also found to be restricted to bud stages [10, 11]. Like FLS from parsley [9], *Matthiola* [10] and *Petunia* [11], the enzyme of carnation is a soluble enzyme which belongs, according to its co-factor requirements, to the 2-oxoglutarate dependent dioxygenases [18]. Moreover, the pH-optimum of the FLS reaction in carnation as well as the results of the inhibitor experiments agree largely with the data reported for the other plants. Thus, we can conclude that in parsley, *Matthiola, Petunia* and carnation the same type of enzyme catalyzes the conversion of dihydroflavonols to flavonols.

In the presence of dominant alleles of the gene R (cyanidin type), part of dihydrokaempferol is hydroxylated in the 3′-position to dihydroquercetin [10] and quercetin are formed in the flowers besides kaempferol derivatives. Consequently, dihydroquercetin was also found to be accepted as substrate for flavonol formation. Interestingly, however, at the anthocyanin level, pelargonidin derivatives are nearly completely replaced by cyanidin derivatives in flowers of genotypes with dominant R alleles [6]. This pattern clearly corresponds to the substrate specificity of FLS and DFR which both use dihydroflavonols as substrates. While for FLS no difference in the rate of the conversion of dihydrokaempferol and dihydroquercetin to the respective flavonols was observed, the DFR enzyme, being involved in anthocyanin synthesis, was found to reduce dihydroquercetin significantly faster (4 times) than dihydrokaempferol [6]. This means that FLS and DFR compete for dihydroquercetin as a substrate, whereas dihydrokaempferol is preferentially converted to kaempferol. Thus, relatively high amounts of kaempferol derivatives are even formed in cyanidin types. Similar observations were made in flowers of *Petunia*. Dihydrokaempferol and dihydroquercetin are comparably good substrates for flavonol formation [11] and the DFR enzyme was found to reduce dihydroquercetin but not at all dihydrokaempferol [19]. On the other hand, the FLS of *Matthiola* prefers dihydrokaempferol [10] and the DFR reduces both dihydrokaempferol and dihydroquercetin [15].

Exact measurements of the most important enzymes involved in flavonoid biosynthesis are a prerequisite for studies on the relationship between the activity of these enzymes, organ development and flavonoid accumulation. The demonstration of FLS activity, the missing link in the enzyme sequence to flavonol formation, has now allowed a more detailed insight into the process of anthocyanin and flavonol formation during bud and flower development.

Four different enzymes were included in this work. Two of them, FLS and DFR, are specifically involved in the formation of flavonols and anthocyanins, respectively. The other two enzymes, CHS and FHT, provide a dihydroflavonol, the common precursor for both flavonols and anthocyanins (see Fig. 1). All of the four enzymes exhibited clear maxima of activity during bud and flower development (Fig. 2a–c). Maximum expression of CHS, FHT and FLS activity is already found in small buds, whereas DFR expression starts late and reaches maximum activity in opened flowers. Comparison of FLS and DFR activities, with the amounts of flavonol and anthocyanin, respectively, at the different developmental stages of buds and flowers reveals a substantial correlation. In

agreement with similar studies in *Matthiola* [10] and *Petunia* [11], the course of FLS activity in carnation shows that flavonol biosynthesis is clearly restricted to the period of bud development, during which no or only a low amount of anthocyanin is present. Because FLS activity already peaks in completely closed buds, induction of this enzyme activity by light can be ruled out. In contrast, DFR activity and anthocyanin formation only occur in opening flowers and may therefore depend on light. Moreover, there is a surprisingly fast switch between the two biosynthetic pathways. FLS activity rapidly declines as soon as anthocyanin synthesis starts. Thus, the activity and accumulation curves provide a differential gene expression during the developmental process of buds and flowers in carnation. By this mechanism the competition of FLS and DFR for dihydroflavonols, in particular for dihydroquercetin, is minimized.

Although FLS activity is drastically diminished in the older developmental stages, the flavonol content of the flowers remains nearly constant indicating that no substantial flavonol degradation occurs in the opening flowers. The anthocyanins formed also seem to be metabolically stable.

The course of the enzyme activities involved in dihydroflavonol formation, CHS and FHT, is similar, but differs from that of FLS and, in particular of DFR. The course is characterized by a rapid increase in the early stages, as also found for FLS, but a rather slow decline of activity in the later stages. This garantees a high supply with dihydroflavonols for flavonol formation in the early developmental stages and a sufficient supply for anthocyanin synthesis in the later stages (Fig. 2a–c). It remains open, whether the course of CHS and FHT activity, respectively, is due to expression of a single gene for each of the enzymes during all developmental stages or whether it is due to the expression of two different genes each for CHS and FHT, one responsible for flavonol formation and the second for anthocyanin synthesis, the latter being possibly induced by light.

Up to now, the enzyme(s) involved in the further conversion of flavan-3,4-diol to anthocyanidin are not known. Considering the course of DFR activity and anthocyanin formation, the demonstration of such enzymes seems to be most promising in protein extracts prepared from stages 6 to 8. On the other hand, early bud stages should be used for the isolation of specific mRNAs for CHS, FHT and FLS, whereas mRNA for DFR and later anthocyanin enzymes can be expected only in opening flowers. Corresponding work is in progress.

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