Planta (1982) 155:176–182

Planta

Chalcone synthesis and hydroxylation of flavonoids in 3′-position with enzyme preparations from flowers of *Dianthus caryophyllus* L. (carnation)

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Abstract. Chalcone synthase activity was demonstrated in enzyme preparations from flowers of defined genotypes of *Dianthus caryophyllus* L. (carnation). In the absence of chalcone isomerase activity, which could be completely excluded by genetic methods, the first product formed from malonyl-CoA and 4-coumaroyl-CoA proved to be naringenin chalcone, followed by formation of naringenin as a result of chemical cyclization. In the presence of chalcone isomerase activity, however, naringenin was the only product of the synthase reaction. In vitro, both 4-coumaroyl-CoA and caffeoyl-CoA were found to be used as substrates for the condensation reaction with respective pH optima of 8.0 and 7.0. The results of chemogenetic and enzymatic studies, however, showed that in vivo only 4-coumaroyl-CoA serves as substrate for the formation of the flavonoid skeleton. In confirmation of these results, an NADPH-dependent microsomal 3′-hydroxylase activity could be demonstrated, catalyzing hydroxylation of naringenin and dihydrokaempferol in 3′-position. Furthermore, a strict correlation was found between 3′-hydroxylase activity and the gene *r* which is known to control the formation of 3′,4′-hydroxylated flavonoid compounds.

Key words: Anthocyanin – Chalcone synthase – *Dianthus* – Flavonoid biosynthesis – Flavonoid-3′hydroxylase.

Introduction

The key enzyme of flavonoid biosynthesis catalyzes the formation of the flavonoid skeleton by stepwise addition of three acetate units from malonyl-CoA to 4-coumaroyl-CoA, or other activated cinnamic acids (Kreuzaler and Hahlbrock 1972). The product of the synthase reaction was previously assumed to be the flavanone and not the isomeric chalcone (Kreuzaler and Hahlbrock 1975). However, chemogenetic and enzymatic studies on chalcone-accumulating mutants of *Callistephus chinensis, Petunia hybrida* and *Dianthus caryophyllus* indicated that the chalcone is the first product of the synthesis of the flavonoid skeleton and that flavanones are formed from chalcones in a stereospecific way, by means of chalcone isomerase (Kuhn et al. 1978; Forkmann and Kuhn 1979; Forkmann and Dangelmayr 1980).

Recent enzymatic investigations on parsley, tulips, and *Cosmos* confirmed this reaction sequence (Heller and Hahlbrock 1980; Sütfeld and Wiermann 1980; Sütfeld and Wiermann 1981). With enzyme preparations of these plants, chalcone synthesis could be demonstrated, but only after total purification of the synthase enzyme from chalcone isomerase activity.

We have now investigated the synthase reaction in crude flower extracts of defined genotypes of *Dianthus caryophyllus,* where chalcone isomerase activity can be excluded by genetic methods. In agreement with the previous chemogenetic and enzymatic studies on chalcone-accumulating mutants of this plant, it is shown that, indeed, the chalcone and not the isomeric flavanone is the first product of the synthase reaction. Furthermore, chemogenetic and enzymologic evidence have established that the formation of 3′,4′-hydroxylated flavanoids is exclusively achieved by a hydroxylation reaction after synthesis of the flavonoid skeleton.

Material and methods

Plant material. This investigation included six defined genotypes of *D. caryophyllus* L. (Table 2). The gene *r* is known to control the hydroxylation pattern of the B-ring of anthocyanins. Recessive genotypes (*rr*) produce pelargonidin derivatives in the flowers, whereas cyanidin is formed under the influence of wild-type alleles

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(*R*) (Geissman and Mehlquist 1947). The gene *a* interferes with the anthocyanin pathway after dihydroflavonol formation, but before anthocyanin synthesis. Recessive genotypes (*aa*) produce white flowers which contain flavonols (Geissman and Mehlquist 1947; Forkmann and Dangelmayr 1980).

In context with our studies the effect reported for the gene *i* is of special interest. This gene is known to control the activity of chalcone isomerase (Forkmann and Dangelmayr 1980). Recessive genotypes (*ii*) lack chalcone isomerase activity and therefore naringenin chalcone 2′-glucoside (isosalipurposide) is accumulated, whereas, because chalcone isomerase activity is present in genotypes with the wild-type allele, higher oxidized flavonoids, including anthocyanins, are synthesized (Fig. 1). The plant material was cultivated in a greenhouse and on the experimental field of our institute.

Chemicals, substrates, and enzymes. Naringenin, apigenin, and dihydroquercetin were obtained from Roth (Karlsruhe, FRG). Eriodictyol, dihydrokaempferol, and isosalipurposide were taken from our laboratory collection. 4,2′,4′,6′-tetrahydroxychalcone was prepared from naringenin according to Moustafa and Wong (1967). 3,4,2′,4′,6′-pentahydroxychalcone was obtained from Sarget (Lyon, France). 4-Coumaroyl-CoA and caffeoyl-CoA were synthesized according to Stöckigt and Zenk (1975) with slight modifications (W. Heller, personal communication). The other activated cinnamic acids, partially purified chalcone synthase and isomerase from parsley, were kind gifts from Dr. W. Heller (Freiburg) and sinapoyl-CoA from Dr. R. Sütfeld (Münster). [2-^{14}C]Malonyl-CoA (2.18 TBq/mol) was obtained from Amersham Buchler (Braunschweig, FRG) and diluted to 0.962 TBq/mol with unlabeled material from Sigma (München, FRG).

[4a,6,8-^{14}C]Naringenin and [4a,6,8-^{14}C]dihydrokaempferol were prepared enzymatically (Forkmann et al. 1980), using partially purified chalcone synthase and chalcone isomerase from parsley.

Enzyme preparation. All steps were carried out at 4° C. One gram flowers were homogenized in a prechilled mortar together with 0.5 g Dowex 1 × 2, 0.5 g quartz sand, and 6.0 ml 0.1 M potassium phosphate buffer, pH 8.0, containing 1.4 mM mercaptoethanol. The homogenate was transferred to Micro Test Tubes (Eppendorf) and centrifugated for 5 min at about 10,000 × *g*. The supernatant was pooled and centrifugated again as described above. The clear supernatant served as the enzyme source for chalcone synthase. Enzyme preparation was also carried out without mercaptoethanol.

Chalcone synthase assay. The standard reaction mixture was based on the assay of Schröder et al. (1979) and contained in a total volume of 100 µl: 60 µl 0.1 M potassium phosphate buffer, pH 8.0 (or pH 7.0), containing 1.4 mM mercaptoethanol, 30 µl crude extract (about 15 µg protein), 5 µl 4-coumaroyl-CoA (1 nmol) (or caffeoyl-CoA), and 5 µl [2-^{14}C]malonyl-CoA (2.44 nmol). After incubation for 3 to 30 min at 30° C, the reaction was stopped by adding 10 µg of the expected chalcones or flavanones in 10 µl methanol. The reaction products were extracted with ethylacetate two times (100 µl + 50 µl). The solution was chromatographed on a cellulose plate with the solvent system 1. The plate was scanned for radioactivity and the radioactive zones were scraped off and counted in 4 ml Unisolve in a scintillation counter. The flavanones formed in assays with chalcone isomerase activity were also measured directly in Unisolve.

Determination of pH optimum. The enzyme assays were carried out in mixtures of 80 µl buffer (between pH 6.0 and 8.3), 10 µl crude extract, and 5 µl 4-coumaroyl-CoA or caffeoyl-CoA, respectively.

Analytical methods. Protein was determined by the method of Bradford (1976). Thin-layer chromatography was performed on precoated cellulose plates (Schleicher & Schüll, Dassel, FRG) with the following solvent systems: (1) chloroform/acetic acid/water (10:9:1, by vol); (2) 30% acetic acid; (3) n-butanol/acetic acid/water (4:1:5, upper phase); (4) acetic acid/HCl/water (30:3:10, by vol). Radioactive zones corresponding to chalcones, flavanones, and dihydroflavonols were scraped off, eluted with methanol, and evaporated to dryness under nitrogen. The residues were redissolved in methanol and co-chromatographed with authentic samples. Furthermore, the residue of the zone corresponding to flavanones was used as substrate for the enzymatic conversion to apigenin, eriodictyol and dihydrokaempferol (naringenin), and dihydroquercetin (eriodictyol), using flower extracts of *M. incana* and *A. majus* (Forkmann et al. 1980; Forkmann and Stotz 1981, Stotz and Forkmann 1981).

Flavonoids were detected under UV-light and after fuming with ammonia. Dihydroflavonols were also detected by the Zinc-HCL test (Barton 1968) and flavanones by reduction with borohydride and subsequent exposure to HCL-fumes (Eigen et al. 1957).

Demonstration of hydroxylases. The preparation of crude extracts was carried out as described above. The enzyme solution was used to demonstrate flavanone 3-hydroxylase activity. The preparation of the microsomal fraction for the determination of the flavonoid 3′-hydroxylase activity, and the assays for both enzymes, were performed according to Forkmann et al. (1980).

Results

General features of the synthase reaction. Incubation of [2-^{14}C]malonyl-CoA and 4-coumaroyl-CoA with crude extracts prepared from the pink, magenta, or white flowering genotype led to the formation of one radioactive product, which was identified as naringenin (*3*) (Fig. 1), by co-chromatography with the authentic flavanone on cellulose thin layer chromatography (TLC) plates in four different solvent systems (Table 1) and by enzymatic conversion to dihydrokaempferol (*5*), eriodictyol (*4*) and apigenin using flower extracts from *Matthiola incana* (Forkmann et al. 1980) and *Antirrhinum majus* (Forkmann and Stotz 1981, Stotz and Forkmann 1981), respectively. When the synthase assay was performed in the presence of the cofactors for the flavanone 3-hydroxylase reaction (2-oxoglutarate, ascorbate, Fe^{2+}), a direct formation of dihydrokaempferol (*5*) was observed, indicating that flavanone 3-hydroxylase activity was also present in the crude extracts. Naringenin was also formed in enzyme assays without mercaptoethanol, but, in the presence of 1.4 mM mercaptoethanol, naringenin synthesis was found to be about three-fold higher. Similar results were reported for the synthase enzyme from flowers of *A. majus* (Spribille and Forkmann 1982).

In contrast to *A. majus* and other plants, however, (Heller and Hahlbrock 1980; Spribille and Forkmann 1981) no by-product was formed with 1.4 mM mercaptoethanol in the enzyme assay. Its formation was

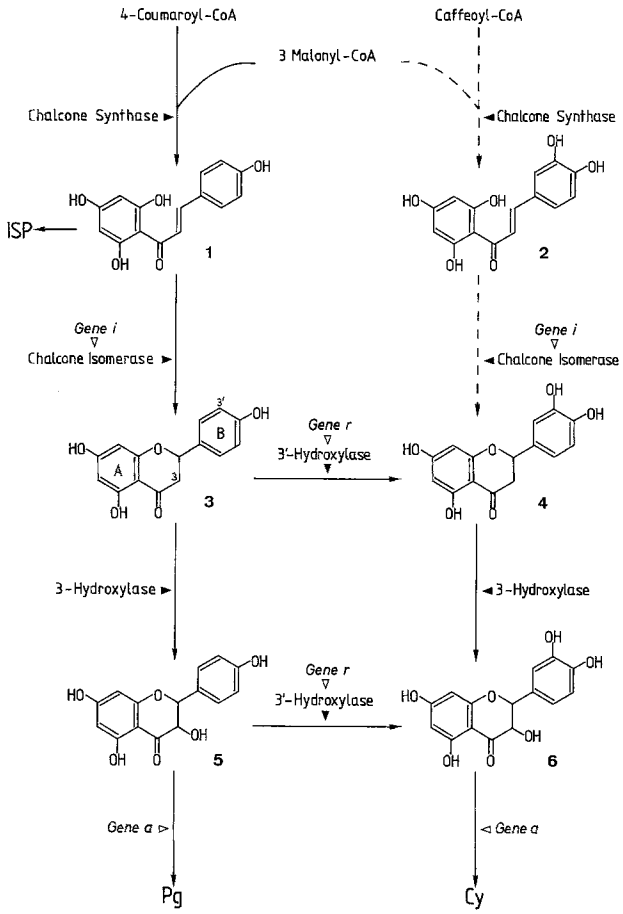

Fig. 1. Genetic control and enzymes of flavonoid biosynthesis in *D. caryophyllus*. The broken lines refer to reactions found only in vitro. Naringenin chalcone (**1**); eriodictyol chalcone (**2**); naringenin (**3**); eriodictyol (**4**); dihydrokaempferol (**5**); dihydroquercetin (**6**). *ISP*, isosalipurposide; *Pg*, pelargonidin; *Cy*, cyanidin

Table 1. R_f-values ($\times 100$) of products on cellulose plates

Compound	Solvent system			
	1	2	3	4
Naringenin chalcone	30	19	93	65
Eriodictyol chalcone	12	16	79	49
Naringenin	83	64	92	89
Eriodictyol	60	56	87	84
Dihydrokaempferol	65	71	90	87
Dihydroquercetin	37	68	85	80

Solvent systems see: Material and methods

only observed at higher mercaptoethanol concentrations. Therefore, the standard assay was carried out in the presence of 1.4 mM mercaptoethanol which warranted a high reaction rate and allowed a direct measurement of naringenin synthesis in the ethyl acetate extracts of the reaction mixture.

Under standard conditions naringenin formation increased linearly with protein concentration up to 15 µg protein per assay. Linearity with time was observed for at least 10 min.

In the presence of 1.4 mM mercaptoethanol, the enzyme preparation could be stored at −20° C for several weeks with a loss of about 25% of activity. Storage in the absence of mercaptoethanol led to a loss of 50% of enzyme activity.

When the enzyme assay was carried out with caffeoyl-CoA instead of 4-coumaroyl-CoA, the reaction product corresponded in four solvent systems to the flavanone eriodictyol (*4*) (Table 1, Fig. 1). The identity of the reaction product with *4* was further confirmed by its enzymatic conversion to dihydroquercetin (*6*) with enzyme preparations from flowers of *M. incana* (Forkmann et al. 1980). Furthermore, incubations of [2-^{14}C]malonyl-CoA and caffeoyl-CoA with crude flower extracts of *D. caryophyllus* in the presence of 2-oxoglutarate, ascorbate, and Fe^{2+} lead directly to the formation of dihydroquercetin.

Naringenin and eriodictyol formation differed markedly in their pH optimum. When 4-coumaroyl-CoA was used as the substrate, the highest formation of naringenin was found at pH 8.0, whereas with caffeoyl-CoA as the substrate the pH optimum for eriodictyol synthesis was at about pH 7.0 (Fig. 2). At the respective pH optima, naringenin formation

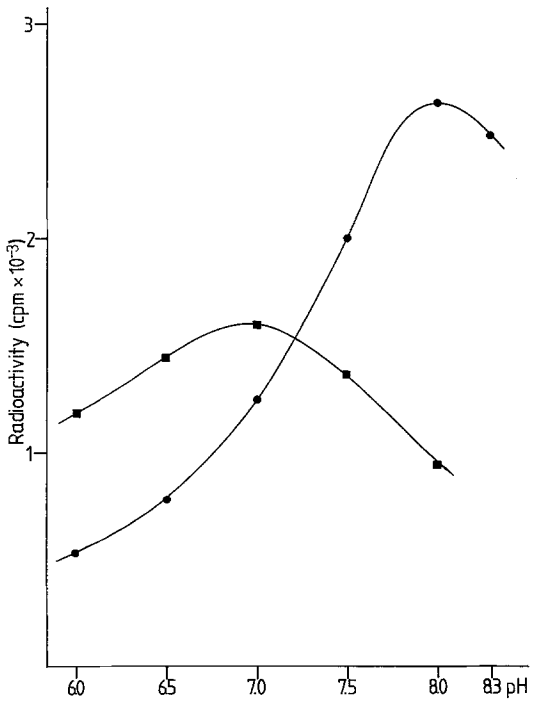

Fig. 2. Dependence of synthase activity on pH. ●—● naringenin formation; ■—■ eriodictyol formation

Table 2. Chemogenetic and enzymatic characterization of six genotypes of *Dianthus caryophyllus*

Genotype	Flower color	Flavonoids	Synthase activity cpm in		Isomerase activity	3′-Hydroxylase activity
			Chalcone[a]	Flavanone[a]		
IIAARR	magenta	cyanidin	0	2,900	present	present
IIAArr	pink	pelargonidin	0	2,850	present	absent
IIaarr	white	kaempferol	0	1,400	present	absent
iiAARR	yellow-magenta	isosalipurposide some cyanidin	1,378	770	absent	present
iiAArr	yellow-pink	isosalipurposide some pelargonidin	5,775	2,844	absent	absent
iiaarr	pure yellow	isosalipurposide some kaempferol	911	507	absent	absent

[a] Naringenin chalcone or naringenin formed with 15 µg protein after 9 min incubation

was about 1.7 times higher than eriodictyol formation. Similar results have been reported for the synthase enzymes from cell cultures of parsley and *Haplopappus gracilis* (Saleh et al. 1978).

Surprisingly, eriodictyol synthesis from caffeoyl-CoA as the substrate could be demonstrated only in the absence of 4-coumaroyl-CoA. In incubations which contained both activated cinnamic acids, only naringenin was found to be formed as a reaction product. Under these conditions eriodictyol synthesis was also not observed at pH 7.0

Cinnamoyl-CoA, feruoyl-CoA, isoferuoyl-CoA, and sinapoyl-CoA were not used as substrates at either pH 8.0 or at pH 7.0 by the synthase enzyme from flowers of *D. caryophyllus*.

Demonstration of chalcone synthesis. Synthase activity could be demonstrated in the crude flower extracts from all six genotypes investigated (Table 2). The products formed from [2-^{14}C]malonyl-CoA and 4-coumaroyl-CoA, however, depended strictly on the genetic state at the locus *i* which is known to control the activity of chalcone isomerase (Forkmann and Dangelmayr 1980). In the genotypes with wild-type alleles (*I*), where chalcone isomerase activity is present, naringenin was found to be the only reaction product (Table 2). But in recessive genotypes (*ii*) which lack chalcone isomerase activity, the formation of a further radioactive product besides naringenin was observed on radiochromatograms of the ethyl acetate extracts from the reaction mixture (Fig. 3a, Table 2). This product was identified as naringenin chalcone (*1*) by co-chromatography with an authentic sample on cellulose TLC plates (Table 1). Incorporation of radioactivity into naringenin chalcone could be readily demonstrated with short incubation times, whereas, with longer incubation, naringenin chalcone was more frequently replaced by the corresponding flavanone naringenin (Fig. 3a). However, when the synthase assay was carried out in the presence of partially purified chalcone isomerase, naringenin was with no exception the only reaction product (Fig. 3b). Naringenin chalcone was also not present in mixed enzyme assays containing crude flower extract from genotypes with wild-type alleles, and those with recessive alleles at the locus *i*.

The individual accumulation curves of naringenin chalcone and naringenin were determined in assays lacking chalcone isomerase activity and compared to the curve of naringenin accumulation observed in the presence of chalcone isomerase activity (Fig. 4). With short incubation times, chalcone formation clearly increased faster than flavanone formation. In agreement with the dependence of the synthase reaction on time, the amount of naringenin chalcone reached a maximum at about 10 min incubation and thereafter declined continuously, accompanied by a corresponding increase in the amount of naringenin. The sum of the individual accumulation curves of the two products agreed largely with the accumulation curve of naringenin, measured in synthase assays with active chalcone isomerase (Fig. 4).

When a crude extract from flowers of genotypes which lack chalcone isomerase activity was incubated with caffeoyl-CoA instead of 4-coumaroyl-CoA, besides eriodictyol (*4*), the formation of eriodictyol chalcone (*2*) was observed. The latter product was identified by co-chromatography with an authentic sample (Table 1). As in the case of naringenin chalcone, incorporation of radioactivity into eriodictyol chalcone increased with the first incubation period, followed by eriodictyol formation as a result of chemical cyclization of the respective chalcone. Furthermore, when chalcone isomerase was added to the synthase assay, even with a short incubation period, eriodictyol was found to be the only radioactive product.

Besides with crude flower extracts of *D. caryophyllus*, chalcone synthesis could also be demonstrated with chalcone isomerase free crude extracts prepared from flowers of chalcone-accumulating mutants of *Callistephus chinensis* (Spribille, in preparation).

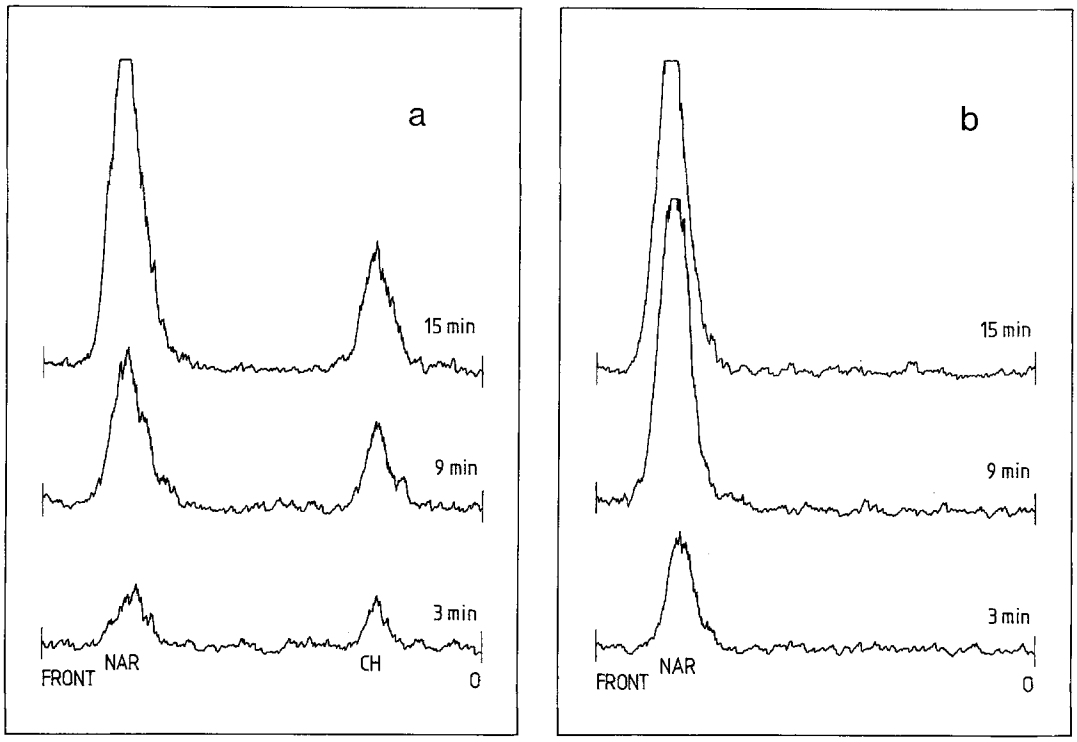

Fig. 3a, b. Formation of naringenin chalcone (CH) and naringenin (NAR) at three different incubation times. **a** Radioscan of thin layer chromatography on cellulose with solvent system 1 from incubations of [2-^{14}C]malonyl-CoA and 4-coumaroyl-CoA with crude flower extracts which lack chalcone isomerase activity. **b** same as (**a**) but with addition of 5 µl partially purified chalcone isomerase to the enzyme assay

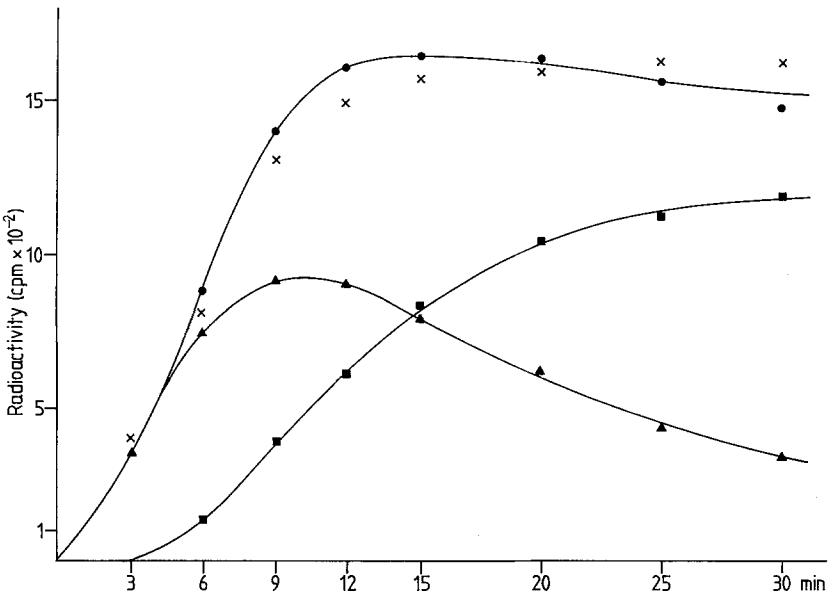

Fig. 4. Time course of incorporation of radioactivity into reaction products. Synthase assay without chalcone isomerase activity: Naringenin chalcone (▲), naringenin (■) and the sum of both (●). Synthase activity with chalcone isomerase activity: Naringenin (×)

Determination of the B-ring substitution pattern. In flowers of *D. caryophyllus*, the locus *r* is known to control the B-ring hydroxylation pattern of anthocyanins. In the presence of the wild-type allele *R*, cyanidin is formed instead of pelargonidin (Table 2). The substrate specificity of the synthase enzyme of *D. caryophyllus* indicated that the substitution pattern of the B-ring of anthocyanins is possibly determined by specific incorporation of either 4-coumaroyl-CoA or caffeoyl-CoA into the flavonoid skeleton with the condensation reaction. But 4-coumaroyl-CoA and caffeoyl-CoA were found to be substrates for the syn-

thase enzyme from both pelargonidin- and cyanidin-producing flowers. Thus, the substrate specificity of the synthase enzyme is obviously not influenced by the locus *r*.

Furthermore, although both 4-coumaroyl-CoA and caffeoyl-CoA can be used as substrates in vitro, the flowers of the genotypes with recessive alleles at the locus *r* were found to contain only 4′-hydroxylated flavonoid compounds (Table 2). Whereas, in the genotypes with wild-type alleles (*R*), 4′- and 3′,4′-hydroxylated flavonoids were formed (Table 2). Independent of the genetic state at the locus *r*, however, naringenin chalcone 2′-glucoside (isosalipurposide) proved to be the only chalcone present in the flowers of genotypes which lack chalcone isomerase activity (Table 2). Eriodictyol chalcone 2′-glucoside was not detected in either the flowers of genotypes with the dominant allele *R* or in the flowers of recessive (*rr*) genotypes. From these results it can be concluded that in *D. caryophyllus* the 3′-hydroxy group is obviously introduced by a specific hydroxylase at the stage of C_{15}-compounds.

A NADPH-dependent microsomal enzyme activity catalyzing hydroxylation of flavonoids in 3′-position was recently found in flower extracts of *M. incana* and *A. majus* (Forkmann et al. 1980; Forkmann and Stotz 1981). It was then possible to demonstrate this enzyme activity in flower extracts of *D. caryophyllus* as well. The enzyme catalyzed hydroxylation of naringenin and dihydrokaempferol in 3′-position to eriodictyol and dihydroquercetin, respectively (Fig. 1). As in *M. incana* and *A. majus*, the 3′-hydroxylase of *D. caryophyllus* was found to be localized in the microsomal fraction and the reaction required NADPH as the cofactor.

Furthermore, enzyme activity proved to be clearly controlled by the locus *r*. Thus, 3′-hydroxylase activity could be readily demonstrated in the flower extracts of all genotypes with the wild-type allele *R*, but was completely deficient in the flower extracts of recessive (*rr*) genotypes (Table 2).

Discussion

The demonstration of the key enzyme of flavonoid biosynthesis, chalcone synthase, in extracts from flowers of higher plants is of special interest. This demonstration allowed, for the first time, investigations of the synthase reaction on a genetically defined plant material. In the course of these studies, we reported recently on successful correlations between the gene *f* (*M. incana*) and the gene *niv* (*A. majus*), respectively, and the activity of chalcone synthase (Spribille and Forkmann 1981; Spribille and Forkmann 1982). However, because the enzyme preparations of these plants also contained chalcone isomerase activity, it remained questionable as to whether the chalcone or the isomeric flavanone was the immediate product of the synthase reaction. We were also able to demonstrate the key enzyme of flavonoid biosynthesis in flower extracts of genetically defined lines of *D. caryophyllus*. This made studies on the products of the synthase reaction formed in presence and in absence of chalcone isomerase activity possible.

Analytical work on flowers of different genotypes of *D. caryophyllus* had shown that in genotypes with recessive alleles at the locus *i* naringenin chalcone 2′-glucoside is accumulated in the flowers, whereas, in the presence of wild-type alleles (*I*), higher oxidized flavonoids including anthocyanins were formed. Furthermore, the chalcone accumulation in the recessive genotypes clearly proved to be caused by a complete lack of chalcone isomerase activity (Forkmann and Dangelmayr 1980). The clear correlation between chalcone accumulation and deficiency of chalcone isomerase activity in recessive genotypes, which was also found in flowers of *C. chinensis* and pollen of *P. hybrida* (Kuhn et al. 1978; Forkmann and Kuhn 1979), demonstrated that the actual product of the synthase reaction is a chalcone rather than a flavanone. In confirmation of the chemogenetic and enzymatic work, chalcone synthesis could then be demonstrated with flower extracts of genotypes of *D. caryophyllus* which lack chalcone isomerase activity. Furthermore, chalcone synthesis was also detected in similar investigations with chalcone isomerase free mutants of *C. chinensis*.

The time course of the synthase reaction shows that in the assays which were totally free from chalcone isomerase activity only the chalcone is synthesized enzymatically, whereas the flavanone is formed from the chalcone by chemical cyclization. In the presence of chalcone isomerase, however, the chalcone formed by the synthase reaction is immediately converted to the corresponding flavanone by chalcone isomerase. These results prove unequivocally that the chalcone is the immediate product of the synthase reaction.

Similar results were recently shown with enzyme preparations from parsley cell cultures (Heller and Hahlbrock 1980), tulip anthers, and flowers of *Cosmos sulphureus* (Sütfeld and Wiermann 1980; Sütfeld and Wiermann 1981). Chalcone synthesis, however, could only be demonstrated with highly purified synthase preparations which were totally free from chalcone isomerase activity. In this connection it should be noted that the present investigations on *D. caryophyllus* and *C. chinensis* could have been performed with crude extracts, because chalcone isomerase activity was simply excluded by genetic methods. Thus, the

use of a genetically defined plant material again proved to be especially adventageous in elucidating flavonoid biosynthesis. Furthermore, using crude extracts of suitable genotypes of *D. caryophyllus*, the in vitro synthesis of naringenin chalcone and eriodictyol chalcone from [2-^{14}C]malonyl-CoA and 4-coumaroyl-CoA or caffeoyl-CoA, respectively, offers a convenient method for obtaining these chalcones in labeled form, in good yield, and with high specific activity. Besides 4-coumaroyl-CoA, caffeoyl-CoA was found to be a suitable substrate for the synthase enzyme of *D. caryophyllus* in vitro. This result indicated that the formation of 3′,4′-hydroxylated flavonoid compounds could be attributed to specific incorporation of caffeoyl-CoA into the flavonoid skeleton. Nevertheless, the chemogenetic investigations on pelargonidin- and cyanidin-producing genotypes showed that caffeoyl-CoA is obviously not used as a substrate in vivo. This assumption is also supported by the fact that in incubations containing both activated hydroxy cinnamic acids only 4-coumaroyl-CoA was used for the condensation reaction. Furthermore, the substrate specificity of the synthase enzyme of *D. caryophyllus* proved to be unaffected by the genetic state at the locus *r* which is known to control the B-ring hydroxylation pattern. In contrast, in investigations on the enzyme flavonoid 3′-hydroxylase, which was found to catalyze hydroxylation of flavonoids in 3′-position in the flowers of *M. incana* (Forkmann et al. 1980) and *A. majus* (Forkmann and Stotz 1981), a strict correlation could be established between the wild-type allele *R* and flavonoid 3′-hydroxylase activity. Both the chemogenetic and enzymologic results prove unequivocally that in flowers of *D. caryophyllus* the formation of 3′,4′-hydroxylated flavonoids is not due to specific incorporation of caffeoyl-CoA during synthesis of the flavonoid skeleton, but is achieved exclusively by a genetically controlled hydroxylation reaction on flavonoid compounds.

These investigations were supported by a grant from the Deutsche Forschungsgemeinschaft. The authors thank Professor W. Seyffert for critical reading of the manuscript.

References

Barton, G.M. (1968) Detection of 3-hydroxyflavanones on papergrams and thin-layer plates. J. Chromatogr. **34**, 562

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye binding. Anal. Biochem. **72**, 248–254

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

Forkmann, G., Dangelmayr, B. (1980) Genetic control of chalcone isomerase activity in flowers of *Dianthus caryophyllus*. Biochem. Genet. **18**, 519–527

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

Forkmann, G., Kuhn, B. (1979) Genetic control of chalcone isomerase activity in anthers of *Petunia hybrida*. Planta **144**, 189–192

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

Geissmann, T.A., Mehlquist, G.A.L. (1947) Inheritance in the carnation, *Dianthus caryophyllus*. IV. The chemistry of flower colour variation. Genetics **32**, 410

Heller, W., Hahlbrock, K. (1980) Highly purified flavanone synthase from parsley catalyzes the formation of naringenin chalcone. Arch. Biochem. Biophys. **200**, 617–619

Kreuzaler, F., Hahlbrock, K. (1972) Enzymatic synthesis of aromatic compounds in higher plants: formation of naringenin from p-coumaroyl-CoA and malonyl-CoA. FEBS Lett. **28**, 69–72

Kreuzaler, F., Hahlbrock, K. (1975) Enzymatic synthesis of an aromatic ring from acetate units. Eur. J. Biochem. **56**, 205–213

Kuhn, B., Forkmann, G., Seyffert, W. (1978) Genetic control of chalcone-flavanone isomerase activity in *Callistephus chinensis*. Planta **138**, 199–203

Moustafa, E., Wong, E. (1967) Purification and properties of chalcone flavanone isomerase from soya bean seed. Phytochemistry **6**, 625–632

Saleh, N.A.M., Fritsch, M., Kreuzaler, F., Grisebach, H. (1978) Flavanone synthase from cell suspension cultures of *Haplopappus gracilis* and comparision with the synthase from parsley. Phytochemistry **17**, 183–186

Schröder, J., Heller, W., Hahlbrock, K. (1979) Flavanone synthase: Simple and rapid assay for the key enzyme of flavonoid biosynthesis. Plant Sci. Lett. **14**, 281–286

Spribille, R., Forkmann, G. (1981) Genetic control of chalcone synthase activity in flowers of *Matthiola incana* R. Br. Z. Naturforsch. **36c**, 619–624

Spribille, R., Forkmann, G. (1982) Genetic control of chalcone synthase activity in flowers of *Antirrhinum majus*. Phytochemistry (in press)

Stöckigt, J., Zenk, M.M. (1975) Chemical synthesis and properties of hydroxycinnamoyl-coenzyme A derivatives. Z. Naturforsch. **30c**, 352–358

Stotz, G., Forkmann, G. (1981) Oxidation of flavanones to flavones with flower extracts of *Antirrhinum majus* (snapdragon). Z. Naturforsch. **36c**, 737–741

Sütfeld, R., Wiermann, R. (1980) Chalcone synthesis with enzyme extracts from tulip anthers tapetum using a biphasic enzyme assay. Arch. Biochem. Biophys. **201**, 64–72

Sütfeld, R., Wiermann, R. (1981) Purification of chalcone synthase from tulip anthers and comparison with the synthase from *Cosmos* petals. Z. Naturforsch. **36c**, 30–34

Received 8 February; accepted 17 March 1982