Phytochemistry, Vol. 21, No. 9, pp. 2231–2234, 1982.
Printed in Great Britain.

0031-9422/82/092231-04$03.00/0

GENETIC CONTROL OF CHALCONE SYNTHASE ACTIVITY IN FLOWERS OF *ANTIRRHINUM MAJUS*

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(*Received* 31 *December* 1981)

Key Word Index—*Antirrhinum majus*; Scrophulariaceae; anthocyanin biosynthesis; chalcone synthase; genetic control.

Abstract—Chalcone synthase activity was demonstrated in flower extracts of defined genotypes of *Antirrhinum majus*. Independent of the genetic state of the gene *eos* which governs the formation of 4'- or 3',4'-hydroxylated flavonoids in the flowers, 4-coumaroyl-CoA was found to be the only suitable substrate for the condensation reaction with malonyl-CoA. Enzyme activity could be detected only in flower extracts of genotypes with wild-type alleles at the locus *niv*. Therefore, the interruption of the anthocyanin pathway in the chalk-white 'nivea' mutant with recessive alleles (*niv*/*niv*) is clearly due to a complete lack of chalcone synthase activity.

INTRODUCTION

Chalcone synthase, the key enzyme of flavonoid biosynthesis, catalyses the formation of the flavonoid skeleton from malonyl-CoA and 4-coumaroyl-CoA or other activated cinnamic acids [1]. The enzyme was first found in enzyme preparations from cell cultures of parsley [1, 2], but could also be detected in different parts of intact plants including flowers [3–7]. Recently, the first successful correlation between the activity of chalcone synthase and a gene in flowers of *Matthiola incana* was reported [8]. In confirmation of analytical work and supplementation experiments, enzyme activity was found in flower extracts of all genotypes with wild-type alleles at the locus *f*, but not in the white-flowering mutants with recessive alleles (*ff*).

We have now investigated the activity of chalcone synthase in flower extracts of different genotypes of *Antirrhinum majus*, where earlier chemogenetic studies [9] and feeding experiments [10, 11] suggested that the action of the gene *niv* is similar to that of the gene *f* in *M. incana*. The results of these studies prove that the interruption of the anthocyanin partway by recessive alleles (*niv*/*niv*) in the chalk-white 'nivea' mutant is clearly due to the lack of chalcone synthase activity and that the formation of 3',4'-dihydroxyflavonoids in the flowers requires the presence of flavonoid 3'-hydroxylase activity.

RESULTS AND DISCUSSION

After incubation of [^{14}C]malonyl-CoA and 4-coumaroyl-CoA with crude flower extracts of the pink genotype (Table 1), the radiogram of the reaction mixture showed two radioactive products in solvent system 1. The main product was identified as naringenin by co-chromatography with the authentic flavanone on cellulose plates in the solvent systems 2 and 3 and by enzymatic conversion to dihydrokaempferol, eriodictyol and apigenin [12, 13]. Furthermore, incubations of [^{14}C]malonyl-CoA and 4-coumaroyl-CoA with crude flower extracts of the pink genotype in the presence of the cofactors for the flavanone 3-hydroxylase reaction (2-oxoglutarate, ascorbate and Fe^{2+}) lead directly to formation of dihydrokaempferol, and in incubations with 1 : 1 mixtures of crude flower extract and microsomal pellet in the presence of NADPH the direct formation of apigenin was observed [13, 17]. In no case could naringenin-chalcone, which is the first product of the synthase reaction (14, 15), be detected, due to the presence of chalcone isomerase in the enzyme preparations. The by-product was not identified; it corresponded in its chromatographic behaviour and other features to the by-product II found in assays of enzyme preparations from flowers of *M. incana* [8]. Thus, it was neither formed in assays with boiled enzyme nor in those without enzyme solution. Furthermore, its formation clearly depended on the concentration of mercaptoethanol during enzyme preparation and in the enzyme assay (Table 2). In contrast to chalcone synthase of parsley [4, 14], the presence of mercaptoethanol was not absolutely necessary for the synthase enzyme of *A. majus*. Naringenin was also formed in assays without mercaptoethanol, whereas the by-product disappeared completely under these conditions (Table 2). This allows the direct measurement of naringenin formation in the ethyl acetate extracts of the reaction mixture, although in the absence of mercaptoethanol the activity of chalcone synthase was somewhat reduced.

Table 1. Genetic control of chalcone synthase activity in different genotypes of *A. majus*

Genotype (homozygous)	Flower colour	Major flavonoid aglycones	Chalcone synthase activity cpm in NAR*
Niv Sulf Inc Eos	Magenta	Ap, Lu, Ch, Qu, Cy	4300
Niv Sulf Inc eos	Pink	Ap, Km, Pg	4600
Niv sulf inc Eos		Ap, Lu, Ch	2800
Niv sulf inc eos	Yellow	Au, Ap	4000
niv Sulf Inc Eos	White	Cinnamic acid glucosides	Not detectable

Au—Aurones, Ap—Apigenin, Lu—Luteolin, Ch—Chrysoeriol, Km—Kaempferol, Qu—Quercetin, Pg—Pelargonidin, Cy—Cyanidin, NAR—Naringenin.
*Product formed with 10 μg protein.

Table 2. Dependence of the formation of naringenin and by-product on mercaptoethanol concentration

Mercaptoethanol (mM)	cpm in naringenin*	cpm in by-product*
none	4983	0
1.4	7048	1087
7.0	7554	2626
14.0	9125	4754

*Product formed with 10 μg protein.

The effect of various inhibitors on formation of naringenin also depended clearly on the concentration of mercaptoethanol in the enzyme assay. In the absence of mercaptoethanol, strong inhibition was found with EDTA, DDC, DPC and especially P-CMB, whereas in the presence of mercaptoethanol (1.4 mM) neither inhibited the synthesis of naringenin (Table 3). In contrast, addition of KCN to the enzyme assay stimulated naringenin formation in each case.

Under standard conditions (1.4 mM mercaptoethanol) the reaction rate was linear with protein concentration up to 5 μg protein/assay and with time to about 30 min. Maximum formation of naringenin was found at about pH 7.0, independent of the presence of mercaptoethanol. Thus, the chalcone synthase from flowers of *A. majus* shows a lower pH optimum than the enzymes from other plants [3–8].

In several plants, other activated cinnamic acids besides 4-coumaroyl-CoA are suitable substrates for the condensation reaction catalysed by chalcone synthase [3, 15]. According to the cinnamic acid starter hypothesis [16], the chalcone synthase of cyanidin-producing genotypes of *A. majus* would be expected to use caffeoyl-CoA instead of 4-coumaroyl-CoA for the formation of the flavonoid skeleton. However, with the enzyme preparation from both pelargonidin- and cyanidin-producing genotypes of *A. majus* 4-coumaroyl-CoA proved to be the only suitable substrate for the condensation reaction. Caffeoyl-CoA was neither used at pH 8.0 nor at lower pH values (pH 7.0 or 6.0), whereas the enzymes from parsley [3] and from flowers of *Verbena hybrida* and *Dianthus caryophyllus* (Spribille, R., unpublished) form eriodictyol readily from caffeoyl-CoA. Furthermore, cinnamoyl-CoA, feruloyl-CoA, isoferuloyl-CoA and sinapoyl-CoA were also not used as substrates by the synthase enzyme of *A. majus*.

The substrate specificity of the synthase enzyme from flowers of *A. majus* is in full agreement with recent enzymatic investigations on hydroxylation reactions involved in flavonoid biosynthesis of this plant [17]. They revealed that the gene *eos* which controls B-ring hydroxylation of flavonoids in *A. majus* clearly controls the activity of flavonoid 3'-hydroxylase. This enzyme catalyses the hydroxylation of naringenin in the 3'-position to eriodictyol. Thus, the formation of 3', 4'-hydroxylated flavonoids including cyanidin in flowers of *A. majus* is not due to the incorporation of caffeic acid into the flavonoid skeleton but is exclusively achieved by a genetically controlled hydroxylation reaction after formation of the flavonoid skeleton.

Earlier chemogenetic studies on *A. majus* have shown that the 'nivea' mutant (*niv*/*niv*) with chalk-white flowers completely lacks flavonoids in the flowers and accumulates hydroxycinnamic acid glucosides [9]. More recently, anthocyanin synthesis has been initiated in the acyanic flowers of this mutant by administration of both dihydroflavonols and flavanones [10, 11]. Thus recessive alleles at the locus *niv* most probably interrupt the anthocyanin pathway at synthesis of the flavonoid skeleton.

Table 3. Effect of various inhibitors on chalcone synthase activity in presence and absence of mercaptoethanol

Additions	Mercaptoethanol 0.0 mM Chalcone synthase activity (%)	Mercaptoethanol 1.4 mM Chalcone synthase activity (%)
none	100	100
1 mM EDTA	54	100
2 mM KCN	136	138
2 mM DDC	28	98
0.5 mM DPC	10	97
0.1 mM P-CMB	5	98

DDC—Diethyldithiocarbamate, DPC—Diethylpyrocarbonate, P-CMB—Chloromercuribenzoate.

Indeed, chalcone synthase activity was observed in flower extracts of genotypes with wild-type alleles at the locus *niv* but was completely absent in floral extracts of the 'nivea' mutant (Table 1). This activity could also not be detected in enzyme preparations from other developmental stages of buds and flowers of the 'nivea' mutant. In mixed enzyme assays containing chalcone synthase and extract from flowers with the recessive allele *niv*, no inhibition of enzyme activity was found. Therefore, the absence of enzyme activity in the 'nivea' mutant is not simply due to the presence of an inhibitor. These results indicate that the gene *niv* controls the activity of this key enzyme of flavonoid biosynthesis.

EXPERIMENTAL

Plant material. The investigations included five genetically defined lines of *Antirrhinum majus* (Table 1). Wild-type alleles of the gene *sulf* are known to suppress aurone formation in the flowers [18]. The gene *inc* controls the activity of flavanone 3-hydroxylase which catalyses hydroxylation of flavanones to dihydroflavonols [17]. The gene *eos* concerns the hydroxylation of flavonoids in 3′-position catalysed by the enzyme flavonoid 3′-hydroxylase [17]. The action of the gene *niv* was of special interest, because recessive alleles most probably interrupt the anthocyanin pathway at an early point [9–11]. The plant material was cultivated in a greenhouse and during the summer in the experimental garden of our institute.

Chemicals and substrates. Naringenin and apigenin were obtained from Roth (Karlsruhe). Eriodictyol and dihydrokaempferol were from our lab collection. 4-Coumaroyl-CoA and caffeoyl-CoA were synthesized according to ref. [19] with slight modifications (Heller, W., personal communication). The other activated cinnamic acids were a kind gift of Dr. Heller (Freiburg) and sinapoyl-CoA from Dr. Sütfeld (Münster). [2-^{14}C]Malonyl-CoA (60 Ci/mol) was obtained from Amersham Buchler and diluted to 26 Ci/mol with unlabelled material from Sigma.

Enzyme preparation. All steps were carried out at 4°C. 1 g flowers were mixed and homogenized in a prechilled mortar together with 0.5 g Dowex 1×2, 0.5 g quartz sand and 6 ml 0.1 M KPi buffer, pH 7.0, containing 1.4 mM mercaptoethanol. The homogenate was centrifuged for 5 min (×2) at about 10 000 g. The clear supernatant served as the enzyme source for chalcone synthase. To achieve a comparison to enzyme preparations without mercaptoethanol the flowers were cut in half lengthwise and the preparation was carried out as described above.

Enzyme assay. The standard reaction mixture contained in total volume of 100 μl: 85 μl 0.1 M KPi buffer (pH 7.0), 1.4 mM mercaptoethanol, 10 μl crude extract (about 5 μg protein), 5 μl 4-coumaroyl-CoA (1 nmol) or other CoA-esters and 5 μl [2-^{14}C]malonyl-CoA (2.44 nmol). After incubation for 5–30 min at 30° the reaction was stopped by adding 10 μg unlabelled naringenin or other flavanones in 10 μl MeOH. The reaction products were extracted with EtOAc (100 μl + 50 μl). The extracts from assays without mercaptoethanol were measured directly in 4 ml Unisolve in a scintillation counter. The extracts from assays with mercaptoethanol were chromatographed on cellulose plates with solvent system 1. The plates were scanned for radioactivity and the radioactive zones were scraped off and counted in Unisolve in a scintillation counter.

Determination of pH optimum. Enzyme assays were carried out in presence and absence of mercaptoethanol (1.4 mM) in mixtures of 85 μl 0.1 M KPi buffer (between pH 6.0 and 8.3) and 5 μl crude extract (pH 7.0).

Analytical methods. Identification of the reaction product and derived compounds and protein determination were carried out according to ref. [8]. TLC was performed on precoated cellulose plates (Schleicher & Schüll) in (1) 15% HOAc; (2) $CHCl_3$–HOAc–H_2O (10 : 9 : 1); (3) 15% EtOH.

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

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