Genetic Control of Chalcone Synthase Activity in Flowers of *Matthiola incana* R. Br.

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Chalcone synthase activity was demonstrated in enzyme preparations from flowers of defined genotypes of *Matthiola incana* (stock). The product formed from 4-coumaroyl-CoA and malonyl-CoA was naringenin and not the isomeric chalcone, because chalcone isomerase was also present in the reaction mixture. Chalcone synthase activity could be detected only in flower extracts of genotypes with wild-type alleles at the locus *f*. Thus, the interruption of the anthocyanin pathway in white flowering lines with recessive alleles (*ff*) of this gene is clearly due to a lack of this enzyme activity. Independent on the genetic state of the locus *b* which controls the formation of pelargonidin or cyanidin, respectively, in the flowers, 4-coumaroyl-CoA was the only suitable substrate for the condensation reaction.

Introduction

Chalcone synthase represents the key enzyme in flavonoid biosynthesis. The enzyme catalyzes the formation of the aromatic ring A of flavonoids by stepwise addition of three acetate units from malonyl-CoA to 4-coumaroyl-CoA [1]. The product of this reaction was previously assumed to be naringenin [2]. But recent enzymatic investigations [3–5] and studies on chalcone accumulating mutants [6–8] revealed that the isomeric chalcone and not the flavanone is the immediate product.

Chalcone synthase activity was first found in enzyme preparations from cell cultures of parsley [1, 2]. More recently, the enzyme could also be demonstrated in different parts of other plants including flowers [4, 5, 9–12]. Furthermore, besides 4-coumaroyl-CoA other activated cinnamic acids were found to be suitable substrates for the condensation reaction [4, 5, 9]. This result indicated that the substitution pattern of the B-ring of flavonoids is possibly determined at the synthesis of the flavonoid skeleton.

For investigations on the genetic control of the chalcone synthase activity and on the substrate specificity of the enzyme we have used flowers of defined genotypes of *Matthiola incana.* Analytical work [13] and supplementation experiments [14] had shown that a white flowering recessive mutant most probably concerns the synthesis of the flavonoid skeleton. We now report on the first successful correlation between the activity of chalcone synthase and the gene *f* of *M. incana.* Enzyme activity was detected only in presence of wild-type alleles. Furthermore, the substrate specificity of the chalcone synthase was found to be uninfluenced by the gene *b* which governs the introduction of the 3′-hydroxy group in the anthocyanins of *M. incana.*

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Materials and Methods

Plant material

The investigations included two cyanic lines and four white flowering mutants of *Matthiola incana* (Table I). The cyanic lines (01 and 09) are only distinguished from each other by the hydroxylation pattern of the anthocyanidins in the flowers, which is determined by the gene *b*. Recessive genotypes (*bb*) produce pelargonidin as aglycone and genotypes with wild-type alleles (b^+b^+) cyanidin (Table I). Chemogenetic investigations [14] and supplementation experiments [13] on the white flowering mutants indicated that the action of the genes *e* and *g* interferes with the anthocyanin pathway after dihydroflavonol synthesis but before anthocyanin formation, whereas the genetic block caused by the recessive allele *f* most probably concerns the formation of the flavonoid skeleton (Fig. 1).

The plant material was cultivated in a greenhouse and during the summer months in the experimental garden of the institute.

Table I. Chemogenetic characterisation of six lines of *M. incana* and the genetic control of chalcone synthase activity.

Line	Genotype	Flower colour	Major phenolic compounds	Chalcone synthase activity
01	$e^+e^+f^+f^+g^+g^+b^+b^+$	bright violet	Cy-glycosides	present
09	$e^+e^+f^+f^+g^+g^+bb$	bright carmin	Pg-glycosides	present
17	$ee\,f^+f^+g^+g^+bb$	white	flavonol glycosides	present
18	$e^+e^+ff\,g^+g^+b^+b^+$	white	hydroxycinnamic acid glycosides	absent
18b	$e^+e^+ff\,g^+g^+bb$	white	hydroxycinnamic acid glycosides	absent
19	$e^+e^+f^+f^+gg\,b^+b^+$	white	flavonol glycosides	present

Chemicals and substrates

[2-^{14}C]Malonyl-CoA (60 Ci/mol) was obtained from Amersham Buchler and diluted to 26 Ci/mol with unlabelled material from Sigma.

4-Coumaroyl-CoA was synthesized according to ref. [15]. The other activated cinnamic acids were a kind gift of Dr. W. Heller (Freiburg).

Naringenin and hesperitin were obtained from Roth (Karlsruhe).

Eriodictyol was prepared by demethylation of hesperitin by a BBr_3 treatment (Kho, personal communication). Homoeriodictyol was isolated from leaves of *Eriodictyon glutinosum* [16].

Enzyme preparation

All steps were carried out at 4 °C. 1 g completely opened petals were homogenized in a prechilled mortar together with 2.0 g Dowex 1 × 2, 0.5 g quartz sand and 6 ml 0.1 M potassium phosphate buffer, pH 8.0, containing 1.4 mM mercaptoethanol. The homogenate was transfered to Micro Test Tubes (Eppendorf) and centrifugated for 5 min at about 10000 × *g*. The supernatants were pooled and centrifugated again as described above. The clear supernatant of the second centrifugation served as enzyme source for chalcone synthase.

Enzyme assay

The reaction mixture contained in a total volume of 100 µl: 85 µl 0.1 M potassium phosphate buffer (pH 8.0), 1.4 mM mercaptoethanol, 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 °C the reaction was stopped by adding 10 µg unlabelled naringenin or other flavanones in 10 µl methanol. The reaction products were extracted with ethyl acetate (200 µl). An aliquote of 50 µl was measured directly in 4 ml Unisolve in a scintillation counter. The remaining solution was chromatographed on a cellulose plate with solvent system 1. The plate was scanned for radioactivity and the radioactive zones were scraped off and counted in Unisolve in a scintillation counter.

Identification of the reaction product

The radioactive zone corresponding to the flavanone on the TLC plate was scraped off, eluted with methanol and evaporated to dryness under nitrogen. The residue was redissoved in methanol and co-chromatographed with authentic flavanones. Furthermore, the residue of the zone corresponding to naringenin was used as substrate for the enzymatic conversion to apigenin, eriodictyol and dihydrokaempferol. The products were identified by the methods described earlier [17, 18].

Determination of pH optimum

Enzyme assays were carried out in mixtures of 85 µl 0.1 M potassium phosphate buffer, 1.4 mM mercaptoethanol (between pH 6.8 and 8.3) and 5 µl crude extract (pH 8.0).

Analytical methods

Protein was determined by the method of Bradford [19]. Thin-layer chromatography was performed on precoated cellulose plates (Schleicher & Schüll) with the following solvent systems: (1) 15% acetic acid; (2) 15% ethanol; (3) chloroform/acetic acid/water (10:9:1, v/v/v). The flavanones were detected under UV-light and by reduction with borohydride and subsequent exposure to HCL fumes [20].

Results

When a crude extract from flowers of line 09 was incubated with [2-^{14}C]malonyl-CoA and 4-coumaroyl-CoA, the radiograms of the reaction mixture showed three products in solvent system 1. The main

Table II. Relative amounts of radioactive products extracted from the reaction mixture and the R_f-values (× 100) on cellulose plates.

Compounds	Amount in %	Solvent systems		
		1	2	3
main product (**I**)	68	41	35	81
naringenin	–	42	35	81
by-product (**II**)	25	92	90	86
by-product (**III**)	7	54	80	66

Solvent systems see: Materials and Methods.

product (**I**) was identified as naringenin by co-chromatography with an authentic sample on cellulose TLC plates in three different solvent systems (Table II). The identity of **I** and naringenin was further confirmed by enzymatic conversion of **I** to apigenin [18], eriodictyol and dihydrokaempferol [17]. The formation of naringenin-chalcone, which is the immediate product of the synthase reaction, was observed neither at 20 min incubation nor at shorter incubation periods.

The identification of the by-products (**II** and **III**) have so far failed. They have been observed also at the investigations with chalcone synthase preparations from parsley cell cultures (Heller, personal communication). But they are not identical with the releasing products described earlier [21]. In assays with boiled enzyme extract or without enzyme solution neither naringenin nor the by-products were found to be present. But **II** is also formed in enzyme assays without 4-coumaroyl-CoA. Furthermore, its formation clearly depends on the mercaptoethanol concentration. At concentrations of more than 14 mM mercaptoethanol, naringenin (**I**) and **III** were found to be completely replaced by **II**. All efforts to exclude the by-products by biochemical methods have so far failed. Therefore, the formation of naringenin could only be measured after chromatographic separation of the extracted reaction products.

The formation of naringenin was linear with time up to 30 min. Linearity with protein concentration was observed only up to 8 µg protein (about 10 µl enzyme extract). The low linearity with protein concentration was clearly due to the low amount of substrates in the standard assay. With higher substrate amounts protein linearity could be substantial extended. Highest formation of naringenin was found between pH 7.5 and 8.0.

It has been reported that chalcone synthase from parsley can be stored under appropriate conditions for several weeks without significant loss of activity [22]. In contrast, the activity of the enzyme from flowers of *M. incana* could be maintained neither in crude extracts nor after precipitation with ammonium sulfate and gel filtration (Sephadex G-25) under the conditions described for parsley. All enzymatic tests were therefore performed with freshly prepared extracts.

Chalcone synthase activity was observed not only in flower extracts of line 09 but also in line 01 and other cyanic lines of *M. incana.* Furthermore, enzyme activity could be demonstrated in flower extracts of the white mutant lines 17 and 19, where the anthocyanin pathway is blocked after dihydroflavonol formation by recessive alleles at the loci *e* and *g* (Table I). In contrast, no formation of naringenin was found with enzyme preparations from flowers of the white mutant lines 18 and 18b with recessive *f*-alleles (Table I). With these extracts, by-product **III** was also not formed, but by-product **II** was found to be still present. Enzyme preparations from other developmental stages of buds and flowers of line 18 were also found to lack chalcone synthase activity. Flavanone formation could also not be achieved

Table III. Substrate specificity of the chalcone synthase prepared from a pelargonidin- or cyanidin-producing line, respectively.

Substrate investigated	Line 09 Genotype: *bb*→Pelargonidin cpm/5 µg protein	Line 01 Genotype: b^+b^+→Cyanidin cpm/5 µg protein	Reaction product
4-coumaroyl-CoA	8975	8430	naringenin
caffeoyl-CoA	1090	577	*
feruoyl-CoA	887	792	*
isoferuoyl-CoA	3052	2988	hesperitin?
cinnamoyl-CoA	655	498	*

* = not identified.

with caffeoyl-CoA or other activated cinnamic acids as substrate.

In context with the question whether the substitution pattern of the B-ring of flavonoids is determined by specific incorporation of definite activated cinnamic acids [23], the substrate specificity of the synthase enzyme was investigated. In flowers of *M. incana*, the locus *b* controls the formation of pelargonidin or cyanidin, respectively (Table I). But the substrate specificity of the chalcone synthase was found to be not influenced by this gene. Thus, 4-coumaroyl-CoA proved to be a suitable substrate for the synthase enzyme prepared from both pelargonidin- and cyanidin-producing genotypes, whereas caffeoyl-CoA was neither used as substrate for the formation of eriodictyol at pH 8.0 nor at pH 6.8 (Table III). At the lower pH value a synthesis of eriodictyol from caffeoyl-CoA was found with enzyme preparations from *Happlopappus gracilis* [9]. The other CoA-esters tested proved to be also poor substrates for the synthase enzyme from flowers of *M. incana*. Only isoferuoyl-CoA yielded small amounts of a product possibly corresponding to hesperitin (Table III).

Discussion

In the last years, flowers of *M. incana* proved to be a valuable source of enzymes involved in flavonoid biosynthesis [17, 25]. Now we could also demonstrate the key enzyme, chalcone synthase,

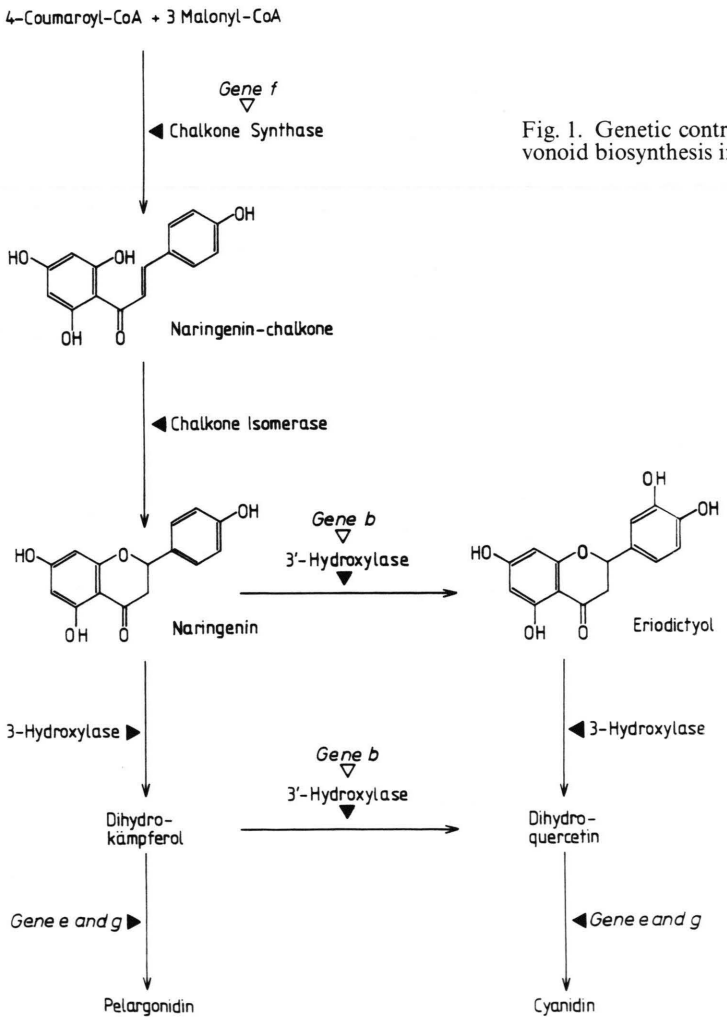

Fig. 1. Genetic control and enzymes of flavonoid biosynthesis in *M. incana*.

which catalyzes the formation of the flavonoid skeleton. Because chalcone isomerase was also present in the reaction mixture, the product formed from malonyl-CoA and 4-coumaroyl-CoA was naringenin and not the isomeric chalcone which is known to be the immediate product of the condensation reaction (Fig. 1). The demonstration of chalcone synthase in flower extracts of genetically defined lines of *M. incana* allowed studies on the genetic control of the enzyme activity and on the possible genetic control of the substrate specificity of the synthase enzyme.

Analytical work on the white flowering mutants had revealed that in presence of recessive *f*-alleles hydroxycinnamic glucosides are accumulated in the flowers [13]. More recently, dihydroflavonols, flavanones and chalcones were found to initiate an anthocyanin synthesis in the white flowers of this special mutant [14]. These results suggested that recessive alleles at the locus *f* most probably interrupt the anthocyanin pathway in flowers of *M. incana* at an early stage. In confirmation of the genetic work, chalcone synthase activity was now detected only on flower extracts prepared from genotypes with wild-type alleles at the locus *f* (Fig. 1). Thus, the interruption of the anthocyanin pathway in the white mutant lines 18 and 18 b by recessive alleles of this gene is clearly due to a lack of the activity of the key enzyme of flavonoid biosynthesis. The correlation between the gene *f* and activity of chalcone synthase proves that the enzyme activity measured in the *in vitro* assays actually catalyzes the synthesis of the flavonoid skeleton *in vivo*. It is now promissing to look for the reason of the deficiency of chalcone synthase activity at the protein biosynthesis and DNA level.

In contrast to the synthase enzyme of other plants [4, 5, 9], the chalcone synthase of *M. incana* was found to use only 4-coumaroyl-CoA as substrate for the formation of the flavonoid skeleton. This high specificity of the synthase enzyme is of special interest in context with the question at which stage of the flavonoid biosynthesis the substitution pattern of the B-ring is determined. In *M. incana* the B-ring pattern of the anthocyanins is controlled by the locus *b*. In the presence of the wild-type allele cyanidin is formed instead of pelargonidin. According to the cinnamic acid starter hypothesis [23], the chalcone synthase from pelargonidin-producing flowers was expected to use 4-coumaroyl-CoA as substrate for the formation of the flavonoid skeleton, whereas for the synthase enzyme from cyanidin-producing flowers caffeoyl-CoA should be the only suitable substrate. But the substrate specificity of the synthase enzyme from flowers of *M. incana* was found to be independent on the genetic state of the locus *b*.

The fact that 4-coumaroyl-CoA is the only suitable substrate for the condensation reaction in *M. incana* is in full agreement with the results of chemogenetic investigations [24]. Furthermore, the gene *b* was recently found to control the activity of flavonoid 3′-hydroxylase which catalyzes the hydroxylation of naringenin and dihydrokaempferol in 3′-position (Fig. 1). These results and the high substrate specificity of the chalcone synthase prove unequivocally that in flowers of *M. incana* the formation of 3′,4′-dihydroxy flavonoid compounds is achieved exclusively by a hydroxylation reaction after synthesis of the flavonoid skeleton.

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