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Characterization and expression of chalcone synthase in different genotypes of *Matthiola incana* R.Br. during flower development

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Abstract

The expression of the key enzyme of flavonoid biosynthesis, chalcone synthase (CHS), has been followed in different genotypes of *Matthiola incana* R.Br. *(Brassicaceae)* which are genetically defined with respect to anthocyanin production. Enzyme activity was determined by a radioactive assay in crude flower extracts. The amount of enzyme protein in the developing flower was determined by use of SDS-PAGE, protein blotting, reaction with an antiserum against CHS of parsley *(Petroselinum hortense),* and PAP staining. The molecular weight of about 41 500 of the CHS subunits corresponds with that obtained from other higher plants. Steps of flower development were subdivided into stages -1,0, I–IV. During flower development of a Matthiola line with coloured petals (line 07) a defined pattern of CHS enzyme production can be observed: At the stage of bud opening (stage 0–I) a dramatic increase of the amount of CHS enzyme protein in the petals occurs. This is quite different from results obtained with petals of the white flowering mutant line 18 bearing a genetic defect in the gene f coding for CHS. Here a reduced and nearly constant level of CHS enzyme protein can be observed during flower development. This line is most attractive for our studies of the regulation of enzyme synthesis because under stress conditions a slight colouring of the flower petals occurs, which is uniformly distributed and line-specific. This suggests that we are dealing with a CHS mutant producing a rather inactive enzyme protein at a low level. This protein may regain enzyme activity under certain environmental conditions. Preliminary investigations suggest a rather high level of CHS mRNA transcription at the bud opening stage of the flowers. Other white flowering mutant lines, line 17 (genotype ee) and line 19 (gg) with a late block in the flavonoid biosynthesis pathway, exhibit a concomitant reduction of CHS enzyme activity and protein content in comparison to anthocyanin-producing lines with the $f^+f^+e^+e^+g^+g^+$-genotype.

Introduction

Regulation of gene expression in higher plants can be studied in the developmentally regulated and/or externally inducible metabolic pathway of flavonoid biosynthesis (4, 7, 1, 29). The enzyme chalcone synthase initially characterized as 'flavanone' synthase in suspension culture cells of *Petroselinum hortense* (9) represents the key enzyme of flavonoid biosynthesis (3, 5). The enzyme catalyzes the stepwise condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA to naringenin chalcone, the central intermediate in the formation of all flavonoid compounds in higher plants (10, 11, 6).

Seyffert (2) demonstrated the usefulness of genetically well defined lines of the stock *Matthiola incana (Brassicaceae)* for the analysis of flavonoids and the characterization of the enzymes involved in anthocyanin biosynthesis and modification. In Matthiola three basic complementary acting genes f, e, and g are necessary for flower pigmentation. Chemogenetic studies and supplementation experiments confirmed that gene f controls the first step of

the flavonoid formation (2). Spribille & Forkmann (25) demonstrated the correlation of gene f and chalcone synthase enzyme activity: white flowering mutant lines of *Matthiola* with recessive alleles at the loci e or g (line 17: genotype ee; line 19: genotype gg; Table 1) showed reduced but detectable enzyme activity in the flower petals (*ca.* 25% of the activity in $f^+f^+e^+e^+g^+g^+$-lines). In the mutant line 18 (genotype ff), also white flowering, no enzyme activity could be detected. However, under field conditions as well as in the greenhouse, slightly pink flowers were observed on line 18. The question arose as to which kind of mutation in the gene f (coding for chalcone synthase) may produce this phenomenon. We therefore investigated the expression of chalcone synthase. The level of enzyme activity and enzyme protein content was determined during flower development and with respect to different genotypes.

Material and methods

Plant material

Matthiola plants (lines 04, 07, 09, and 17, 18, 19) were cultivated in the field or in the greenhouse of the Lehrstuhl für Genetik, Tübingen (Table 1).

Flower development was subdivided in several stages from –1 to IV, each representing a distinct step of morphological development:

–1: Completely closed and very small bud. The uncoloured petals are not visible.

0: Nearly closed bud. The top of the uncoloured petals becomes visible.

I: Nearly closed bud. The top of the petals becomes visible.

II: The petals are intensively coloured but unfolded.

III: The petals begin to unfold.

IV: The petals are opening to build a 90° angle.

Table 1. Genotype and phenotype of the used *Matthiola* lines.

Line	Complementary acting genes for flower pigmentation			Anthocyanin modifying genes				Type of anthocyanin
	f	e	g	b	l	u	v	
04	+	+	+	+	+	+	v	Cyanidin
07	+	+	+	+	+	u	v	Cyanidin
09	+	+	+	b	+	+	+	Pelargonidin
17	+	e	+	+	+	+	+	-
18	f	+	+	+	+	+	+	- (± cyanidin)
19	+	+	g	+	l	u	+	-

Absorption spectra

Methanol (1% HCl) extracts of flower petals (stage III–IV) were obtained by shaking 0.5 g fragmented petals (fresh weight) in 1 ml MeOH/HCl 24 h at 4 °C in the dark. After centrifugation (3 000 **g**; 10 min) absorption spectra of the supernatants were determined against a MeOH/HCl reference for wave-lengths between 250 and 800 nm in an Uvikon 820 spectrophotometer.

CHS enzyme activity

CHS enzyme activity was determined in *Matthiola* petals of stage III–IV which were frozen with liquid nitrogen and stored at –20 °C up to use. Crude extracts were obtained by homogenization at 4 °C of 0.25 g petals (fresh weight) with 0.5 g quartz sand, 1 g Dowex 1 × 2, and 3 ml 0.1 M potassium phosphate buffer pH 7.5 (1.4 mM β-mercaptoethanol) in a mortar (modified after 25). After two centrifugation steps (each 10 000 **g**; 5 min) CHS enzyme activity was determined in the last supernatant: the enzyme assay contained 45 µl crude extract, 5 µl 4-coumaroyl-CoA (final concentration 1×10^{-5} M), and 5 µl (2-^{14}C)malonyl-CoA (26 Ci/mol; final concentration 2.44×10^{-5} M), and potassium phosphate buffer in total 100 µl (modified after 10). After 30 min at 30 °C ^{14}C-labelled naringinin was determined in ethyl acetate according to (25).

Immunoprecipitation of CHS

CHS was immunoprecipitated by the method of Kreuzaler *et al.* (11). 45 µl crude petal extract (see above), 0.1 M potassium phosphate buffer pH 7.5 (1.4 mM β-mercaptoethanol), and different volumes of dialyzed rabbit antiserum against *Petroselinum* CHS (11) were mixed in a total of 90 µl. Control assays contained unspecific rabbit serum of identical protein content instead of CHS antiserum. After 45 min at 26 °C, 4–5 h at 6 °C, and

centrifugation (10000 g; 10 min) CHS enzyme activity was determined in the supernatant.

Identification of CHS enzyme protein in Matthiola petals by electroblotting, immunoprecipitation, and PAP-staining

Crude protein extracts were obtained by homogenization of petals with 0.1 M potassium phosphate buffer pH 7.5 (1.4 mM β-mercaptoethanol) at 4 °C in a mortar as follows:

A: 0.25 g (fresh weight) frozen petals
0.5 g quartz sand
1.2 ml buffer

B: 2.5 g petals
1.0 g quartz sand
2.0 ml buffer

C: Extraction B and concentration in Sephadex G-100 (20)
After the concentration step these extracts were brought to a volume of 600 µl

Proteins were denatured and then separated on 10% SDS-polyacrylamide gels (110 × 90 × 1.5 mm) with 3% stacking gels (13) at 70 V (*ca.* 70 mA; 30 min) and 20 V (*ca.* 20 mA; 16 h). Marker proteins for molecular weight determinations were α-lactalbumin (subunit M_r = 14 400), soybean trypsin inhibitor (20 100), carbonic anhydrase (30 000), ovalbumin (43 000), bovine serum albumin (67 000), and phosphorylase b (94 000). They were visualized by Serva Blue G-staining.

The electrophoretic transfer of proteins to nitrocellulose (modified after 27) was done at 6 °C in transfer buffer (0.025 M Tris, 0.192 M glycin, 20% v/v methanol) between two steel plates (18 × 20 cm; 0.4 A; 1.5 h; according 17).

The localization of CHS enzyme protein on the nitrocellulose filters was possible by use of the PAP-method (26). Therefore the nitrocellulose filters were shaken sequentially at room temperature in

1. 100 ml PBS (0.1% BSA, 0.05% Triton X-100); 1.5 h
2. 100 ml PBS (1% BSA); 3 h
3. 5 ml rabbit antiserum against *Petroselinum* CHS, 1:500 diluted in PBS (0.1% BSA); 16 h
4. 4 × 100 ml PBS (0.1% Triton X-100); 1 h
5. 5 ml goat antiserum against rabbit IgG (GAR), 1:100 diluted in PBS (0.1% BSA); 1 h
6. = 4
7. 5 ml peroxidase antiperoxidase (PAP), 1:250 diluted in PBS (0.1% BSA); 1 h
8. = 4

Finally the filters were incubated in a solution containing 3.3 ml 4-chloro-1-naphthol (3 mg/ml ethanol), 16.7 ml 0.1 M Tris-HCl pH 7.5, and 10 µl 30% H_2O_2. A staining reaction was visible within a few minutes.

Protein was determined by the method of Lowry *et al.* (15). Coloured extracts were first treated with Dowex 1 × 2 (2 × 0.1 g/400 µl).

Chemicals

(2-^{14}C)Malonyl-CoA (60 Ci/mol) was obtained from Amersham Buchler and diluted to 26 Ci/mol with unlabelled material from Sigma. PAP and GAR were obtained from Miles. Rabbit antiserum for *Petroselinum* CHS (11) was a kind gift of Prof. K. Hahlbrock (Köln). Unspecific rabbit serum was obtained from Prof. W. Bessler (Tübingen).

Results

Chalcone synthase enzyme activity

CHS enzyme activity was measured by a radioactive assay in crude protein extracts of flower petals of *Matthiola* line 07 (genotype f^+f^+) in comparison to line 18 (genotype ff). Table 2 shows the results of determining enzyme activity immediately or 5–6 h after preparation of the crude extract. For line 07 a clear decrease in enzyme activity occurred within 5 h whereas for line 18 the already low radioactivity detectable in the ethyl acetate phase stayed at the same background level (about 2×10^{10} cpm/kg protein). This result confirmed earlier observations that no CHS enzyme activity is detectable in petals of line 18. Furthermore no naringenin could be

Table 2. CHS enzyme activity in crude extracts of flower petals.

Line	Determination of enzyme activity	Enzyme activity (cpm/kg protein)
07	Immediately	$1 - 4 \times 10^{11}$
	5–6 h later	$7 \times 10^{10} - 3 \times 10^{11}$
18	Immediately	$1 - 2 \times 10^{10}$
	5–6 h later	2×10^{10}

found by thin layer chromatography (24). Inhibition of CHS enzyme activity in the assay by immunoprecipitation was possible with a rabbit antiserum for parsley CHS (11). Radioactivity was reduced specifically after mixing antiserum with 07-flower extracts (Fig. 1). A complete inhibition of enzyme activity could be observed with increasing amounts of antiserum. In control experiments an unspecific rabbit serum had no effect on CHS enzyme activity. In extracts of line 18 the low background radioactivity decreased slightly after the addition of CHS antiserum but the same effect was observed after the use of the unspecific rabbit serum – suggesting an unspecific reaction (Fig. 2). This

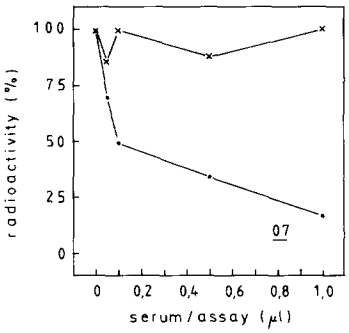

Fig. 1. Immunoprecipitation of *Matthiola* CHS in crude extracts of 07-petals with rabbit antiserum for *Petroselinum* CHS (●—●). Enzyme activity of non-precipitated CHS was determined in a radioactive assay (100% = 2.2×10^{11} cpm/kg protein). In the control (×—×) unspecific rabbit serum instead of CHS antiserum was added to the extracts. Data were obtained from three independent experiments.

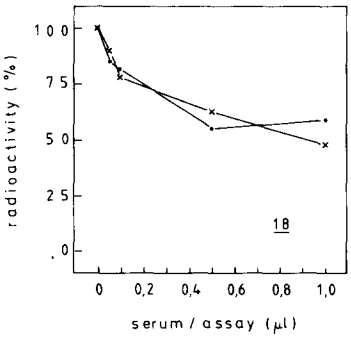

Fig. 2. Evidence for CHS inactivity in *Matthiola* line 18. Crude extracts of petals were treated as described for Fig. 1. (●—●) addition of CHS antiserum; (×—×) addition of unspecific rabbit serum. (100% = 3.8×10^{10} cpm/kg protein).

experiment showed that with the indirect method of immunoprecipitation no enzyme activity in 18-petals can be found.

Characterization of CHS enzyme protein

CHS enzyme protein was detected in crude extracts of *Matthiola* petals. The methods used were SDS-PAGE, protein blotting on nitrocellulose, reaction with antiserum for parsley CHS, and PAP-staining. Figure 3 shows the specific reaction of the CHS subunits from lines with genotype $f^+f^+e^+e^+g^+g^+$. One distinct band is visible at a molecular weight of about 41 500 (values ranging from 39 500 to 43 000 in 42 determinations). Separation of parallel extracts (same g fresh weight) of the white flowering mutant lines 17 (genotype ee) and 18 (genotype ff) revealed enzyme protein in these lines, too (Fig. 4). In several comparable experiments lines 17 and 19 (genotype gg) showed only *ca.* 3% of the CHS protein content of the $f^+f^+e^+e^+g^+g^+$-lines. Surprisingly line 18 has even more CHS enzyme protein, in the range of about 10% of the $f^+f^+e^+e^+g^+g^+$-plants. These results are summarized in Fig. 5. The latter result was rather unexpected as line 18 proved to have the genetic block at the chalcone synthase level (23, 2) and showed no enzyme activity. In addition, there is no

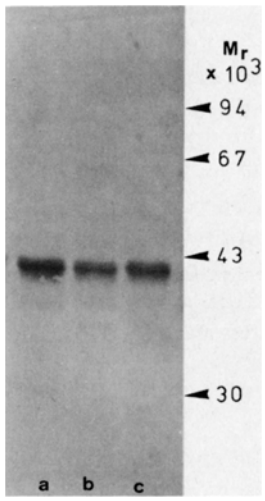

Fig. 3. CHS enzyme protein in crude extracts from petals of line 04 (lane a), 07 (b), and 09 (c) (extract preparation A; ca. 300 mg protein/lane). This figure shows a photograph of a nitrocellulose filter after PAP-staining. Proteins were separated by gel electrophoresis on 10% SDS-PAG.

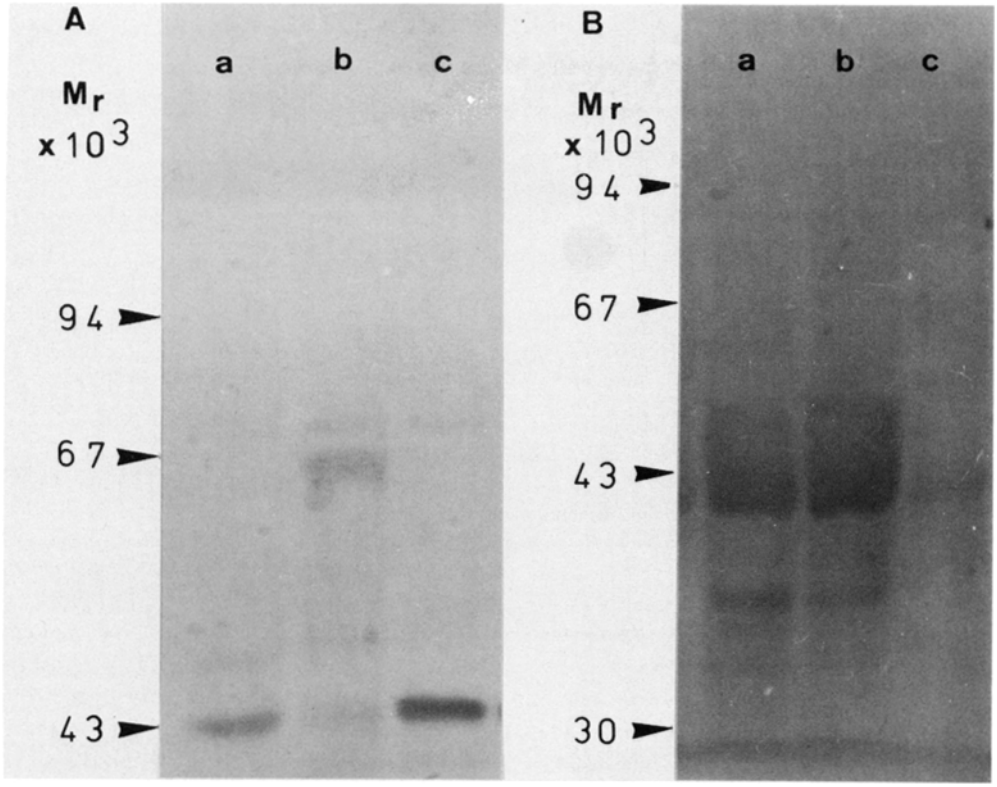

Fig. 4. CHS enzyme protein in crude extracts of petals of the *Matthiola* mutants 17 and 18.
A: Line 18 (white petals; lane a), line 17 (b), and a control extract (line 09; c); extract preparation A.
B: Line 18 (pink petals; lane a), line 18 (white petals; b), and line 17 (c); extract preparation C.

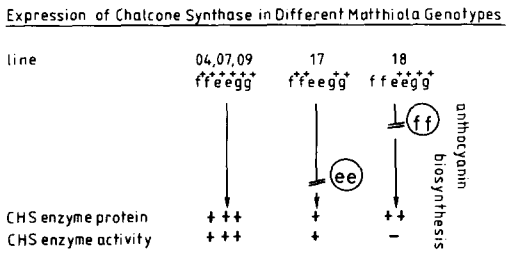

Fig. 5. Compilation of data on CHS enzyme protein and enzyme activity in genetically defined Matthiola lines. Symbols: (+++) high, (++) lower, (+) low, (-) no CHS enzyme activity or enzyme protein, respectively, measured at stage II-III of flower development (relative values).

obvious difference in the amount of CHS protein in white 18-petals in comparison to slightly pink petals of line 18 (Fig. 4B). In these pink petals anthocyanin from the line-specific cyanidintype can be spectrophotometrically determined. Figure 6 shows the absorption spectra of methanol-HCl extracts of petals from line 18 (white flowers), 18 (pink flowers), and the cyanidin-producing line 07.

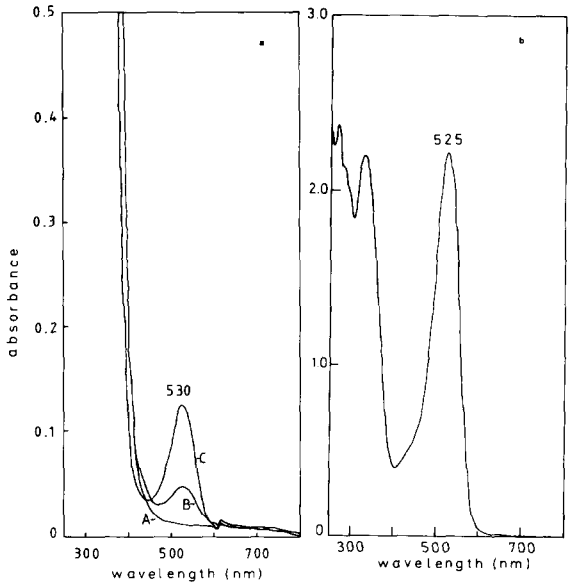

Fig. 6. MeOH/HCl spectra of extracts from petals of a) line 17 (A), 18 (B; slightly pink), and 18 (C; pink), and b) of the obligatory cyanidin producing line 07 (genotype $f^+f^+e^+e^+g^+g^+$. The 07-extract was prepared of 0.2 g petals in 2 ml MeOH/HCl and 1:10 diluted before scanning.

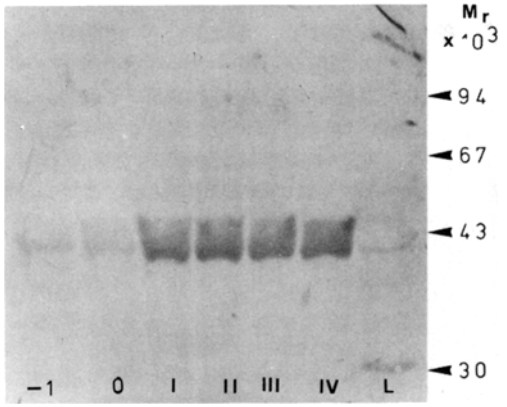

Fig. 7. CHS enzyme protein in *Matthiola* petals of different developmental flower stages (-1–IV) and in leaves (L) of line 07 (preparation A). Methods used as described for Fig. 3.

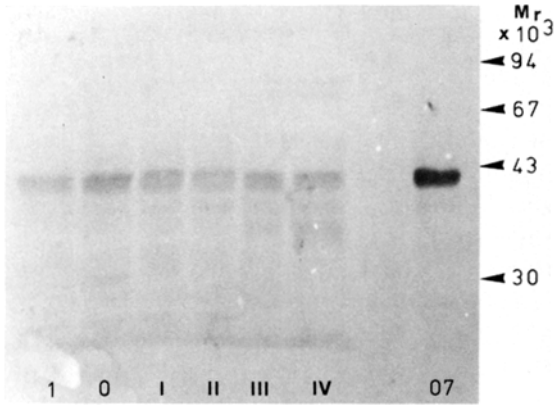

Fig. 8. CHS enzyme protein in *Matthiola* petals of different developmental flower stages of line 18 (preparation A; petals of stage II–IV were slightly pink). Methods used as described for Fig. 3.

Expression of chalcone synthase during flower development

During flower development of *Matthiola* the activity of enzymes involved in the biosynthesis and in the modification of anthocyanins shows a characteristic time course (1). Therefore, we wanted to see whether there is a specific, developmentally regulated, stage where the *de novo* synthesis of CHS enzyme protein occurs. Crude protein extracts from 0.25 g petals (fresh weight) of different developmental stages (-1 to IV) were separated by SDS-PAGE and localized after protein blotting as described above. Figure 7 shows for line 07 (genotype $f^+f^+e^+e^+g^+g^+$) that closed buds with uncoloured petals contain only a small amount of CHS enzyme protein which increases considerably between stage 0 and I and stays apparently constant thereafter (stage I–IV).

In petals of stage -1 of the mutant line 18 (Fig. 8) a similar amount of CHS enzyme protein is detected in comparison to stage -1 of line 07, and this level remains constant during further flower development. If one takes into account the decline in total protein content per g fresh weight from stage I to IV (Fig. 9) only a slight increase of CHS enzyme protein per mg total protein can be observed.

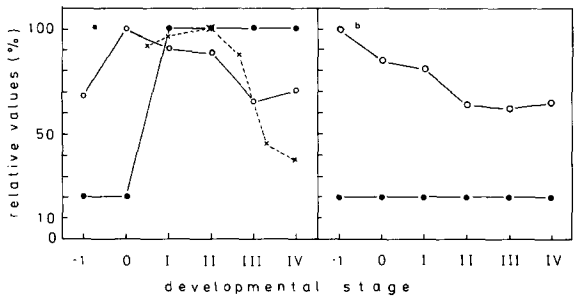

Fig. 9. Expression of CHS during flower development of *Matthiola* lines 07 (a) and 18 (b). For CHS enzyme protein level (●—●) compare Figs. 7 and 8 (100% = level in stage I-petals of line 07). CHS enzyme activity data (×- - - -×) were taken from (1). Total protein content (07: 100% = 4.0 mg/ml crude extract; 18: 100% = 4.2 mg/ml crude extract) as well as CHS enzyme protein content were calculated on fresh weight basis (○—○).

Discussion

Chalcone synthase in *Matthiola* uses 4-coumaroyl-CoA exclusively as substrate in the stepwise condensation reaction with malonyl-CoA leading to the first intermediate of flavonoid biosynthesis, naringenin chalcone (2, 25). The decision as to which type of anthocyanin is produced depends on the presence of gene b coding for the flavonoid 3′-hydroxylase (23) which modifies ring B.

Cross reaction of an antiserum specific for parsley chalcone synthase (11) made it possible to characterize independently the activity and the total amount of CHS in different genotypes of *Matthiola*. Special interest was focussed on line 18 carrying a genetic block in gene f, which obviously does not produce the chalcone but in which flavonoid syn-

thesis can be obtained by the addition of naringenin (19). Interestingly, sometimes, under stresslike conditions, a slight but uniform colouring of flower petals occurs. In these line 18-petals a low amount of cyanidin is measured. In contrast to these findings, so far no CHS enzyme activity could be found, either by direct radioactive assays (as already shown by 25) or by the indirect immunoprecipitation method described in this paper.

The molecular weight of the CHS subunits of about 41 500 is in the range found for several plant suspension cultures, where values of 42 000 (*Petroselinum hortense,* 11), 42 500 (*Phaseolus vulgaris,* 14), 41 000 (*Glycine max,* 8), and 42 000 (*Petunia hybrida,* 16) have been determined. All these data were obtained with antiserum against the parsley enzyme. The molecular weight of the complete *Petroselinum* enzyme, consisting of two identical polypeptides, was found to be about 77 000 (11). The specificity of the CHS antiserum for our experiments was shown by competitive inhibition of CHS enzyme of line 07-petals with increasing amounts of antiserum.

Synthesis of flavonoids is possible in all parts of the plant. Extensive production can occur in petals of developing flowers. Correspondingly, in leaves of *Matthiola* (Fig. 7), but mainly in flower petals (stage I–IV), of all anthocyanin producing genotypes, CHS enzyme protein is found after protein separation, blotting, and PAP-staining.

In the white flowering mutant lines 17 (genotype ee), 18 (ff), and 19 (gg), each with a genetic block in one of the complementary acting genes involved in flower pigmentation, CHS enzyme protein was detected by the methods mentioned above. In line 17 and 19 where the block is located after the formation of the dihydroflavonols (2), CHS enzyme activity is reduced to about 25% of the values obtained with $f^+f^+e^+e^+g^+g^+$-lines (Forkmann, personal communication). Correspondingly, a low amount of enzyme protein is produced. In comparison to lines 07 or 09 in lines 17 or 19 reduction to a level of 3% is estimated. This suggests a regulation mechanism which reduces the CHS protein level *and* the CHS enzyme activity in the non-anthocyanin synthesizing mutants 17 and 19.

In line 18, with a defect in the CHS gene f, a higher level of enzyme protein is present compared with the other white flowering mutant lines 17 and 19. There is no difference between white and pink flowers of line 18. This shows that the CHS gene in line 18 is expressed but leads to a protein with no detectable enzyme activity.

In *Matthiola* CHS activity follows a certain developmentally regulated time course (1). We could show that the step of bud opening (here: stage I) is associated with an enormous increase of CHS protein in the $f^+f^+e^+e^+g^+g^+$-lines. The drastic increase in enzyme formation is accompanied by an increase in enzyme activity (Fig. 9A and 1). Whereas the enzyme activity decreases in older petals the enzyme protein level remains constant, suggesting an inactivation of active enzyme protein (Fig. 9A) which does not alter the molecular weight significantly. After the UV-induced *de novo* synthesis of active enzymes involved in flavonoid biosynthesis in parsley cell suspension cultures the inactivation of a previously active CHS was determined (21). A similar process happens in the pigmented *Matthiola* flower petals (genotype $f^+f^+e^+e^+g^+g^+$) during flower development. In line 18 the level of CHS protein does not change considerably during flower development (Figs. 8 and 9A). Interestingly, we have evidence that on the level of RNA transcription the CHS gene is most active at stage I–II both in f^+f^+-lines as well as in line 18 (unpublished results).

We can only speculate on the nature of the ff-mutation at the moment. We know that line 18 exhibits a genetically inherited defect of CHS. Gene f is mutated, but a gene product cross reacting with a CHS specific antiserum is synthesized. However, this expression does not lead to an enzyme active under normal conditions. The result is a white flower. Under certain exogenous and/or endogenous conditions (stress!) the flower petals show a weak but uniform cyanidin pigmentation. A transposon-like phenomenon as described for specific mutants of *Antirrhinum majus* (28) can, therefore, probably be excluded. Reimold *et al.* (18) supply evidence for a second CHS-like gene in *Petroselinum hortense,* which might be also developmentally regulated under normal conditions. If there is such a second CHS gene also in *Matthiola* one could conceive that its gene product replaces the defective CHS in line 18, thus leading to weak anthocyanin production.

For the conditional mutant of *Petunia hybrida* Red Star (16) a different mechanism has to be assumed, because no CHS gene product is found in

the white coloured sectors of the petals. Since we know CHS mRNA is synthesized in line 18 (unpublished results) and since CHS protein is present a more probable explanation for the kind of mutation can be proposed: Line 18 produces rather an inactive CHS protein under normal conditions which might possibly be able to regain enzyme activity under stress conditions. The assumed difference between the wild type and the mutant enzyme may be located in a small region (catalytic center) and is under further investigation. We are currently making further studies in the genetically well defined *Matthiola* system to compare wild type and mutant chalcone synthase at the level of gene structure and regulation of transcription.

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