Phytochemistry, Vol. 26, No. 4, pp. 991–994, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00
Pergamon Journals Ltd.

GENETIC CONTROL OF HYDROXYCINNAMOYL-COENZYME A: ANTHOCYANIDIN 3-GLYCOSIDE-HYDROXYCINNAMOYLTRANSFERASE FROM PETALS OF *MATTHIOLA INCANA*

MONIKA TEUSCH, GERT FORKMANN and WILHELM SEYFFERT

Universität Tübingen, Institut für Biologie II, Lehrstuhl für Genetik, Auf der Morgenstelle 28, D-7400 Tübingen, F.R.G.

(*Revised received* 4 *July* 1986)

Key Word Index—*Matthiola incana*; Cruciferae; anthocyanin biosynthesis; acylated anthocyanins; acyltransferase; genetic control; HPLC.

Abstract—Anthocyanidin 3-glucosides and 3-sambubiosides acylated with 4-coumarate or caffeate were identified in flower extracts of lines of *Matthiola incana* with wild-type alleles of the gene **u**. An enzyme activity was demonstrated catalysing the acylation of 3-glucosides and 3-sambubiosides with 4-coumarate or caffeate using the respective CoA esters as acyl donors. Anthocyanins glycosylated in both the 3- and 5-positions were not acylated. The enzyme exhibited an pH optimum at 6.5 and was inhibited by divalent ions, EDTA, diethylpyrocarbonate and *p*-chloromercuribenzoate. Accumulation of acylated 3-glycosides during bud development is correlated with acyltransferase activity. In confirmation of the chemogenetic work, acyltransferase activity was found only in enzyme extracts from flowers of lines with the wild-type allele $\mathbf{u}^+$.

INTRODUCTION

Genes controlling the acylation of anthocyanins have been identified in a range of plants [1–6]. Until now, however, an enzyme catalysing this reaction was only demonstrated in *Silene dioica* [6]. This enzyme catalysed the transfer of the coumaroyl or caffeoyl moiety from the respective CoA esters to anthocyanidin 3-rhamnosylglucoside and 3-rhamnosylglucoside-5-glucoside. The activity of the acyltransferase is controlled by the gene Ac of *Silene*.

In flowers of *Matthiola incana*, differently acylated 3-glycosides and 3,5-glycosides of pelargonidin and cyanidin have been found [5]. The formation of these anthocyanins is governed by the genes **l**, **u** and **v**, which modify the anthocyanidin 3-glucosides present in the flowers of genotypes with recessive alleles of these three genes. Gene **l** is known to control the activity of anthocyanin 5-*O*-glucosyltransferase, leading to the formation of 3,5-glycosides [7]. The genes **u** and **v** obviously concern the acylation of the anthocyanins with 4-hydroxycinnamic acid derivatives [5]. No acylated anthocyanins have been found in flowers of lines with recessive alleles. The fact that large amounts of acylated anthocyanidin 3-sambubiosides are restricted to lines with wild-type allele $\mathbf{u}^+$ led to the conclusion that gene **u** controls both xylosylation and acylation [5]. We now report on the presence of acylated 3-glucoside and 3-sambubioside in flowers of a line with the wild-type alleles $\mathbf{u}^+\mathbf{u}^+$ and on the demonstration of an enzyme activity which catalyses the acylation reaction and which is controlled by the gene **u**.

RESULTS

Line 08 with recessive alleles of the anthocyanin modifying genes **l**, **u** and **v** contains mainly cyanidin 3-glucoside (Cy 3-G) and some cyanidin 3-xylosylglucoside (sambubioside = Cy 3-B) in flowers. Line 06 with dominant alleles of gene **u** and recessive alleles of the genes **l** and **v** contains the anthocyanins listed in Table 1. These anthocyanins were identified by TLC in three solvents, by HPLC, controlled acid hydrolysis and spectral data. The acylated anthocyanins showed the normal spectral maxima of the cyanidin glycosides, with additional peaks between 315 and 325 nm indicating acylation with hydroxycinnamic acid. Alkaline hydrolysis was performed in order to determine the nature of the glycoside and the acyl residue. Authentic samples of these anthocyanins were later separately provided by enzymatic acylation of the unacylated precursors (see below).

The fact that in flower extracts of line 06 acylated cyanidin 3-glucosides and 3-sambubiosides are present led to enzymatic studies of acylation with 3-glucosides and 3-sambubiosides as substrates. When enzyme preparations from flowers of line 06 were incubated with 4-coumaroyl-CoA and cyanidin 3-glucoside, the latter compound was found to be acylated with 4-coumarate.

Table 1. Anthocyanin content of petals from *Matthiola incana* R. Br. line 06 (HPLC analysis)

Anthocyanin	Retention time (min)
Cy 3-G	3.9
Cy 3-B	3.3
Cy 3-(caffeoyl)-B	5.3
Cy 3-(4-coumaroyl)-B	5.6
Cy 3-(caffeoyl)-G	5.6
Cy 3-(4-coumaroyl)-G	5.9

Moreover, cyanidin 3-sambubioside as substrate was acylated to the same extent as the 3-glucoside. Identification of both acylated anthocyanins was achieved with authentic compounds by HPLC, TLC in different systems, alkaline hydrolysis and by spectral analysis. When caffeoyl-CoA was used as the acyl donor, the respective caffeates of cyanidin 3-glucoside or 3-sambubioside were formed. The retention times of the products formed from cyanidin 3-glucoside with 4-coumaroyl- and caffeoyl-CoA and cyanidin 3-sambubioside with the same CoA esters corresponded with those present in the flower extracts of *Matthiola incana* line 06 (Table 1). With 4-coumaroyl-CoA as the acyl donor and cyanidin 3-sambubioside, 15 nmol of acylated anthocyanin was formed within 10 min incubation at 25°. In contrast, the reaction rate with caffeoyl-CoA as the acyl donor was weaker (about 15%). Feruloyl-CoA, however, was a poor donor (about 10%) and with sinapoyl-CoA, isoferuloyl-CoA, cinnamoyl-CoA and 4-methoxycinnamoyl-CoA the reaction failed completely. Aliphatic acid-CoA-esters such as acetyl-, malonyl-, succinyl- or glutaryl-CoAs also did not serve as acyl donors.

Tests with enzyme preparations from other genotypes revealed that enzyme activity for acylation of 3-glucoside and 3-bioside is only present in lines with the wild-type allele $\mathbf{u}^+$. Line 08 and other genotypes with recessive alleles of the gene **u** completely lack this enzyme activity. To exclude the possibility that in lines with recessive (**uu**) alleles soluble inhibitors suppress the acylation reaction, mixtures from genotypes with acylation activity and without this enzyme activity were prepared. No inhibition of enzyme activity was observed.

Moreover, acylation was found to be independent of the gene **l** and surprisingly also of the gene **v**, which had been supposed to be involved. Thus no enzyme activity was observed in genotypes with the wild-type allele $\mathbf{v}^+$ and recessive alleles of the gene **u**. The acylation reaction was further characterized using cyanidin 3-sambubioside and 4-coumaroyl-CoA as substrate and Sephadex G-50 eluates of the crude extracts from line 06 as enzyme source. Passing the crude extract through Sephadex G-50 removed contaminating anthocyanins which interfere with the quantitative analysis of the reaction mixture by HPLC. In contrast to the acyltransferase of *Silene dioica* [6], the enzyme of *Matthiola incana* lost no activity during this procedure. Product formation was found to be proportional to the amount of added enzyme up to 30 μg Linearity with time was observed up to 15 min. Maximal activity was exhibited at pH 6.5. The pH values for half-maximal activities were 6.0 and 7.7, respectively. An increase of enzyme activity was obtained from 0° up to 30°. But even at 0° enzyme activity was present (10%). The calculated E_a between 0 and 10° amounted to 70 kJ/mol, from 10 to 30° it increased to 91 kJ/mol, and above 30° it decreased to 11 kJ/mol. The acylation was saturated with 30 nmol of Cy 3-B and it was strongly inhibited when the substrate concentration exceeded the value of 90 nmol. When the Cy 3-B concentration was increased to above 90 nmol, the enzyme was progressively inhibited. Saturation with 4-coumaroyl-CoA was achieved with 15 nmol; even a ten-fold increase in concentration did not affect the production of acylated anthocyanins.

The acylation reaction is rather sensitive to inhibitors such as *p*-chloromercuribenzoate, diethylpyrocarbonate, and diethyldithiocarbamate or divalent ions. With the exception of Ca^{2+} and Mg^{2+}, the reaction is strongly inhibited by inorganic ions (Table 2). No ions were found to stimulate the reaction. This is in good accordance with the results of *Silene dioica* [6].

In addition to cyanidin 3-glucoside and 3-sambubioside, a range of other anthocyanins were tested as substrates. Pelargonidin 3-glucoside, the respective sambubioside and delphinidin 3-glucoside, which does not occur in flowers of *Matthiola*, were found to be acylated with similar rates to those of the cyanidin derivatives. But cyanidin 3,5-diglucoside and 3-sambubioside-5-glucoside were not used as substrates with 4-coumaroyl-CoA, caffeoyl- or sinapoyl-CoA. Different combinations of the genes **u** and **v** also did not change these results.

The course of acyltransferase activity as well as acyl anthocyanin accumulation in flowers was studied during bud and flower development. Morphological criteria were used to divide the development process into eight stages [8]. As Fig. 1 shows, the amount of acyl anthocyanin is low in buds, but increases rapidly to a maximal level between stages 4 and 5, where it remains for the last three stages. In good correlation with the amount of acyl anthocyanins, the acyl transferase activity starts from a low level at stage 1 and increases rapidly to a maximum between states 4 and 5. In enzyme preparations of the following stages the enzyme activity decreases rapidly.

DISCUSSION

In confirmation of the chemogenetic work [7–11], acylation activity was present only in flower extracts from lines of *Matthiola incana* with the wild-type allele $\mathbf{u}^+$. Moreover, the substrate specificity of the enzyme is in full accord with the anthocyanin pattern found in the flowers of line 06 with wild-type alleles of gene **u**. The lack of activity in flower extracts with recessive (**uu**) alleles proves that the enzyme activity measured *in vitro* is really responsible for acylation *in vivo*. This is further confirmed by the close correlation between enzyme activity and accumulation of acylated anthocyanins during bud and flower development.

The acyltransferase from flowers of *Matthiola* is similar to the enzyme from *Silene* in being inhibited by divalent

Table 2. Influence of divalent ions and inhibitors on acyltransferase

Addition	Concentration (mM)	Total activity (%)
None	1	100
$MgCl_2$	1	90
$CaCl_2$	1	98
$MnCl_2$	1	89
$CaCl_2$	1	44
$FeSO_4$	1	14
$CuSO_4$	1	19
$ZnSO_4$	1	16
$ZnCl_2$	1	18
EDTA	1	77
KCN	1	82
Diethylpyrocarbonate	1	89
Diethyldithiocarbamate	2	69
P-Chloromercuribenzoate	0.2	37

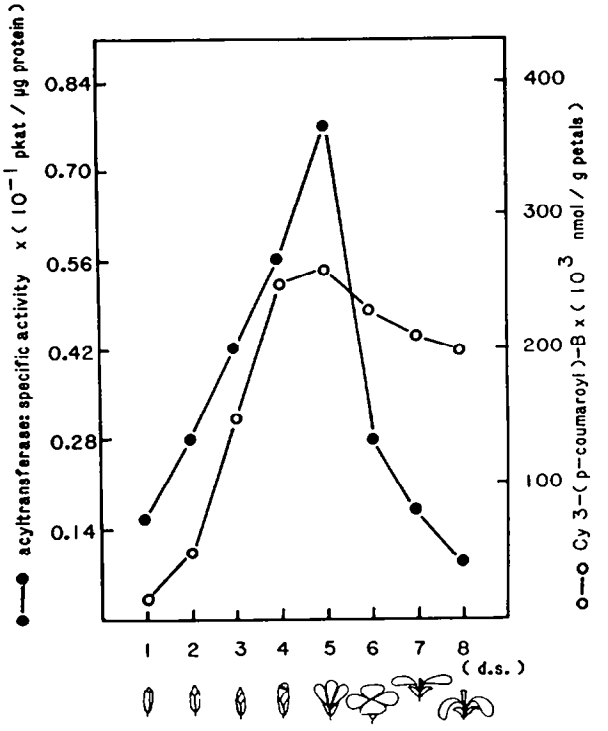

Fig. 1. Activity of the acyltransferase and accumulation of acylated cyanidin 3-sambubioside [Cy 3-(4-coumaroyl)-B] during bud and flower development. d.s., Development stage.

metal ions, and both enzymes use 4-coumaroyl- and caffeoyl-CoA as acyl donors. However, the enzymes behave differently towards EDTA and *p*-chloromercuribenzoate, exhibit different pH optima and use different substrates. In *Silene dioica* [6], acylated 3-rutinoside is not an appropriate substrate for enzymatic 5-glucosylation but 3-rutinoside-5-glucoside is a suitable substrate for acylation. In contrast, flower extracts of *Matthiola incana* readily convert acylated 3-sambubioside to the respective 5-glucoside [7] but do not use this compound or 3,5-diglucoside as a substrate for acylation. Thus, in *Silene*, acylation of 3,5-glycosides seems to be the final step in anthocyanin biosynthesis whereas in *Matthiola* acylation obviously precedes 5-glucosylation.

Former chemogenetic results suggest a pleiotropic action of the gene **u** governing acylation and xylosylation. Since acylation is clearly controlled by this gene, the acylation reaction is either a prerequisite for xylosylation, which is controlled by an as yet unknown gene, or gene **u** is a regulation gene which controls the expression of enzyme activity for acylation as well as xylosylation. Recent studies on an enzyme catalysing xylosylation support the first hypothesis (M. Teusch, in preparation).

As yet, no acylating activity has been detected in lines with recessive alleles of the gene **u** although wild-type alleles of the gene v^+ should also be involved in acylation of anthocyanins [5]. The idea that the 3,5-glycosides are the true substrates for the acylation governed by the gene **v** has therefore to be rejected. It is possible that gene **v** is responsible for the formation of doubly acylated anthocyanins [5, 12]. Further work will be concerned with the elucidation of this question.

EXPERIMENTAL

Plant material. The investigations included the cyanic lines 01–08 of *Matthiola incana* R. Br. [7]. They were cultivated in a greenhouse during summer in the experimental garden of our institute.

Chemicals and references. The anthocyanins pelargonidin, cyanidin and delphinidin 3-glucosides, pelargonidin and cyanidin 3-sambubioside, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside-5-glucoside and acylated cyanidin 3-sambubioside were isolated from methanolic flower extracts by descending PC (Whatman) in BAW and 30% HOAc. The aliphatic CoA esters were purchased from Sigma (St. Louis, MO). The hydroxycinnamic CoA esters 4-coumaroyl-, caffeoyl-, feruloyl-, isoferuoyl-, cinnamoyl- and sinapoyl-CoA were gifts from W. Heller, Freiburg.

Enzyme preparation. 1.0 g petals was homogenized in a prechilled mortar together with 1.0 g quartz, 0.5 g PVP (Serva) and 6 ml 0.1 K-Pi buffer, pH 7.0, containing 5 mM 2-mercaptoethanol at 4°. The homogenate was centrifuged for 10 min at 10000 *g*. The supernatants were pooled and again centrifuged under the described conditions. The crude extract was passed through a Sephadex G-50 column (bed vol. = 1 ml) to free it from phenolic compounds and other low-molecular-weight substances.

Enzyme assay. The standard reaction mixture consisted of 20 µl enzyme (20 µg protein), Cy 3-sambubioside (30 nmol), 20 µl 4-coumaroyl-CoA (20 nmol) and 60 µl 0.05 M K-Pi buffer, pH 7.0. The reaction mixture was incubated for 10 min at 25° and stopped by addition of 50 µl $CHCl_3$–MeOH (0.5% HCl) (2:1), resulting in a Folch partition [13]. The anthocyanins were concentrated in the upper phase. This phase was analysed by HPLC and TLC.

Analytical methods. For HPLC analysis, 50 µl of the upper phase was injected into a HPLC (Spectra Physics SP 8700) equipped with a Spherisorb ODS II (5 µm, RP 18) column (50 × 4.6 mm) and a guard column (10 × 4.6 mm) filled with the same material (Bischoff, Leonberg). Detection was performed at 530 nm with a variable wavelength detector (Knauer). Separation was accomplished by a linear gradient elution. The linear gradient was changed from 20 to 70% A portion in A + B = 100 (A = 1.5% H_3PO_4 in MeCN, B = 0.5% H_3PO_4 in H_2O) in 5 min, then within 2 min from 70 to 100%. Analysis was performed at a flow rate of 1.5 ml/min and at room temp. The retention times and quantitative calculations were obtained with a computing integrator (Spectra Physics SP 4270). To obtain definite identification of the acylated anthocyanins, the reaction mixture and the fractions of the HPLC separation were charged with the appropriate references on cellulose plates (Schleicher & Schüll) in BAW. UV-VIS spectra and alkaline hydrolysis were carried out for further identification [12].

Protein estimation. The protein content of the enzyme preparation was estimated by the method of Bradford, with bovine serum albumin as standard [14].

Acknowledgement—These investigations were supported by grants from the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Timberlake, C. F. and Bridle, P. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds) pp. 214–266. Chapman & Hall, London.
2. Hrazdina, G. (1982) in *The Flavonoids: Advances in Research* (Harborne, J. B. and Mabry, T. J., eds) pp. 133–188. Chapman & Hall, London.
3. Wiering, H. and de Vlaming, P. (1973) *Genen Phaenen* **16**, 35.

4. Jonsson, L. M. V., Aarsman, M. E. G., van Diepen, J., de Vlaming, P., Smit, N. and Schram, A. W. (1984) *Planta* **160**, 341.
5. Seyffert, W. (1960) *Z. Pflanzenzücht.* **44**, 4.
6. Kamsteeg, J., van Brederode, J. and van Nigthevecht, G. (1980) *Biochem. Physiol. Pflanz.* **175**, 403.
7. Teusch, M., Forkmann, G. and Seyffert, W. (1986) *Planta* **168**, 586.
8. Teusch, M., Forkmann, G., and Seyffert, W. (1986) *Z. Naturforsch. Teil C* **42**, 699.
9. Spribille, R. and Forkmann, G. (1981) *Z. Naturforsch. Teil C* **36**, 619.
10. Forkmann, G., Heller, W. and Grisebach, H. (1980) *Z. Naturforsch. Teil C* **35**, 411.
11. Heller, W., Britsch, L., Forkmann, G. and Grisebach, H. (1985) *Planta* **163**, 191.
12. Harborne, J. B. (1964) *Phytochemistry* **3**, 151.
13. Folch, J., Less, M. and Saane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 496.
14. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.