Anthocyanins Isolated from Petals of Various Genotypes of the Red Campion *(Silene dioica (L.) Clairv.)*

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Anthocyanin pigments present in petals of various genotypes of *Silene dioica* were identified by R_F-values, UV-spectra, partial and total hydrolysis, alkalysis, NMR- and IR-spectra. Both the 3-glucosides, 3,5-diglucosides, 3-rutinosides and 3-rutinosides-5-glucosides of cyanidin and pelargonidin were found.

The location of the acyl group at the 3-O-bound sugar in acylated anthocyanins — *p*-coumaric acid in pelargonidin-glycosides and caffeic acid in cyanidin-glycosides — has been determined by partial hydrolysis, peroxide oxidation, and oxidative cleavage with sodium periodate followed by reduction with sodium borohydride and identification of the polyalcohols formed after hydrolysis. The phenolic acids have been shown to be attached to the 4-hydroxyl group of rhamnose in anthocyanidin 3-rhamnosylglucosides and to the 6-hydroxyl group of the 3-O-bound glucose in anthocyanidin 3-O-glucosides.

Introduction

In *Silene dioica* normally variation in anthocyanins does not occur. By means of hybridization with *S. alba*, inbreeding and selection, a large variation in petal-color and, therefore in anthocyanins can be found.

Interest in the flower-color inheritance in crosses between *S. dioica* and *S. alba* dates back to Bartram [1]. Mendel also studied the inheritance of flower-color in the offspring of these crosses and Onslow [2] already suggested the presence of both apigenin- and cyanidin-derivatives in petals of the offspring. Baker [3], who studied the autecology of *S. dioica*, *S. alba* and their hybrids reported that in addition to cyanidin-diglucosides also malvidin-diglucosides were present in petals of *S. dioica*.

To provide a sound foundation for the genetical studies on flower color inheritance and for the biochemical studies on glycosylation, hydroxylation and acylation in *Silene*, the elucidation of the structure of the anthocyanins in the various genotypes was carried out.

Much emphasis has been given to anthocyanin structure and location of the acyl group in individual acylated anthocyanins isolated. Little is known about the structure of acylated anthocyanins and the genetics of acylation. We hereby report the elucidation of the structure of several acyl-anthocyanins isolated from petals of various genotypes.

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Results and Discussion

Examination by paper chromatography of anthocyanins isolated from petals of *Silene dioica* plants, which were selected from various European populations, revealed only two anthocyanin spots (tri and tri-ac). The main compound tri-ac possesses apart from the typical absorption peaks of the anthocyanin-spectrum also a peak at 310 – 330 nm, and is easily converted into tri. This peak at 310 – 330 nm points to acylation with a cinnamic acid derivative. After purification by two-dimensional paper chromatography, compound tri-ac had a ratio $E_{300-330}/E_{\text{vis max}}$ of 0.46, indicating that the anthocyanin tri-ac has only one acyl group (Harborne [4]).

From the presence of an inflection at 440 nm in the UV-spectrum of both the compounds tri and tri-ac it can be concluded that the 5-hydroxyl position is substituted (Jurd, Geissman *et al.* [5, 6]).

The presence of ortho di-OH groupings in the B-ring was indicated by a bathochromic shift in the absorption maxima of both tri and tri-ac in the presence of aluminium chloride (Jurd *et al.*, Harborne and Polock *et al.* [7 – 9]).

Aglycone: After total hydrolysis the same aglycone and sugars were obtained from both tri and tri-ac. From the R_F-values of the aglycone in various

solvents and from the spectrophotometric data it could be concluded that the aglycone was cyanidin (see experimental). This was confirmed by co-chromatography with authentic cyanidin. Partial hydrolysis of tri yielded cyanidin, cyanidin 3-glucoside, cyanidin 3-rhamnosyl(1 → 6)glucoside, and of tri-ac also cyanidin 3-(rhamnosyl(1 → 6)glucoside)-5-glucoside(tri).

Sugars liberated: The liberated sugars could be identified as glucose and rhamnose by co-chromatography on TLC with several sugar references. The molar ratio, determined according to the method of Pridham [10] was 2 : 1 (glucose-rhamnose).

Peroxide oxidation followed by alkaline hydrolysis according to the method of Chandler and Harper [11] of tri yielded rutinose (α-L-rhamnosyl (1 → 6)-β-D-glucose), which was chromatographically and spectroscopically indistinghuishable from an authentic sample of rutinose, prepared by peroxide oxidation of rutin.

Acyl compound: Changes in the R_F-values and absorption spectrum after alkaline hydrolysis of the compound tri-ac confirmed that the pigment was acylated. The acyl moiety obtained by alkaline hydrolysis was chromatographically and spectroscopically indistinghuishable from caffeic acid (see experimental). Both the acyl moiety and caffeic acid were visible as grey spots after spraying with *p*-nitroaniline, and as tan spots with diazotized sulfanilic acid (Krebs *et al.* [12]). Both caffeic acid

Table I. Compounds liberated after various degradative techniques of the three main acylated anthocyanins present in petals of *S. dioica*.

Formed products	UV-spectra	R_F	Color	Technique used
A. d-ac				
d-ac	279, 329, 535	0.28	red-purple	none
cy 3 G (m)	277, 538	0.38	red-purple	partial acid hydrolysis
cy 5 G	278, 540	0.39	red-purple	partial acid hydrolysis
cyanidin	277, 546	0.69	red-purple	total acid hydrolysis
6-caffeyl-β-D-glucose	250, 333	0.51	tan *	H_2O_2-peroxide oxidation
glucose	206, 251	0.15	tan *	total acid hydrolysis
caffeic acid	230, 285, 315	0.80	—	alkaline hydrolysis
B. tri-ac				
tri-ac	279, 329, 535	0.33	red-purple	none
cy 3 RG 5 G (tri)	278, 535	0.24	red-purple	partial acid hydrolysis
cy 3 RG (rhgl)	277, 538	0.37	red-purple	partial acid hydrolysis
cy 3 G (m)	277, 538	0.38	red-purple	partial acid hydrolysis
cyanidin	277, 546	0.69	red-purple	total acid hydrolysis
4-caffeyl-α-L-rhamnosyl(1 → 6) β-D-glucose	207, 244, 335	0.42	brown *	H_2O_2-peroxide oxidation
4-caffeyl-α-L-rhamnose	226, 335	0.80	yellow-grey *	partial hydrolysis Dowex
rutinose	206, 258	0.09	brown *	partial hydrolysis Dowex
glucose	206, 251	0.15	tan *	total acid hydrolysis
rhamnose	208, 250	0.31	yellow-grey *	total acid hydrolysis
caffeic acid	230, 285, 315	0.80	—	alkaline hydrolysis
C. pg-tri-ac				
pg-tri-ac	271, 313, 517	0.38	orange-red	none
pg 3 RG 5 G (pg-tri)	272, 518	0.30	orange-red	partial acid hydrolysis
pg 3 RG (pg-rhgl)	271, 518	0.37	orange-red	partial acid hydrolysis
pg 3 G (pg-m)	272, 517	0.44	orange-red	partial acid hydrolysis
pelargonidin	270, 530	0.82	orange-red	total acid hydrolysis
4-*p*-coumaryl-α-L-rhamnosyl (1 → 6)-β-D-glucose	207, 244, 313	0.55	brown *	H_2O_2-peroxide oxidation
4-*p*-coumaryl-α-L-rhamnose	225, 313	0.78	yellow-grey *	partial hydrolysis Dowex
rutinose	206, 258	0.10	brown *	partial hydrolysis Dowex
glucose	206, 251	0.15	tan *	total acid hydrolysis
rhamnose	208, 250	0.32	yellow-grey *	total acid hydrolysis
p-coumaric acid	223, 290, 311	0.90	—	alkaline hydrolysis

UV-spectra of anthocyanins were recorded in ethanol, 0.01% hydrochlorid acid. R_F-values were determined on Whatman I paper developed in the solvent system *n*-butanol-acetic acid-water (4 : 1 : 5, v/v/v, upper phase). * Color after spraying with aniline hydrogenphthalate. See legend Table II.

and the acyl compound had identical NMR- and UV-spectra and decomposed after the addition of sodium hydroxide (Ribéreau-Gayon [13]).

Location of acyl group: Peroxide oxidation according to Chandler and Harper [11] of the purified anthocyanin tri-ac yielded a caffeylrutinoside (see Table I). Partial hydrolysis of the caffeylrutinoside with acetic acid or of the acyl-anthocyanin tri-ac with Dowex W-X 8 (H^+-form) (Watanabe [14]) liberated in both cases β-D-glucose and a caffeylrhamnoside showing the acyl group to be attached to the rhamnose molecule.

To determine the location of the acyl group at the rhamnose molecule, compound tri-ac was oxidized with sodium periodate, followed by reduction with sodium borohydride and hydrolysis with hydrochloric acid (Watanabe *et al.*, Metzenberg *et al.* [14, 15]). This treatment yielded two polyalcohols, which were identified as glycerol and 1,2,3-butanetriol by co-chromatography in *BAW* and ethyl acetate-pyridine-water (7 : 2 : 1, v/v/v). The reference 1,2,3-butanetriol was prepared from crotylalcohol by the method of Lieben [16]. The polyalcohols were made visible by spraying with potassium metaperiodate, respectively followed by spraying with a borax-starch solution (Metzenberg [15]).

The liberation of glycerol is typical for a glucose molecule, of which either the 1- or the 1- and 6-position are occupied (5-O- and 3-O-bound glucose of the anthocyanin respectively). 1,2,3-Butanetriol is formed from a rhamnose molecule of which both the 1- and the 4-position are occupied (Watanabe *et al.* [14]). From this it can be concluded that the caffeyl group is bound at the 4-position of the rhamnose molecule. This was confirmed by NMR-spectroscopy of the purified caffeylrutinoside obtained by hydrogen peroxide oxidation of compound tri-ac (see experimental). Therefore compound tri-ac is: cyanidin 3-(4-caffeyl-α-L-rhamnosyl(1 → 6)-β-D-glucoside)-5-β-D-glucoside and compound tri: cyanidin 3-(α-L-rhamnosyl(1 → 6)-β-D-glucoside)-5-β-D-glucoside.

Anthocyanins present in petals of various genotypes

In a population of *S. dioica* in Limburg, Netherlands, a plant was found which had, contrary to the normal reddish-purple, pink petals. It appeared that this color was formed when a gene called *P* was present in homozygous recessive state. By crossing with *S. alba* and selection for both "P" and "p", plants with a large variation in anthocyanins were found in the offsping.

Several genes causing this variation could be identified. Apart from genes controlling the amount of anthocyanin present, the genes *P*, *M*, *N* and *Ac* appeared to be responsible for structural variation in the anthocyanin molecule, as shown by differences in chromatographic behaviour. In *P/P* plants the anthocyanins present in the different *M*, *N* and *Ac* gene combinations were reddish-purple, whereas in *p/p* plants only orange-red anthocyanins were present.

(i) Reddish-purple flowers. From petals of reddish-purple flowers of *P/P* plants with different *M*, *N* and *Ac* gene combinations eight different reddish-purple anthocyanins were isolated. By means of acid hydrolysis it could be demonstrated that all these anthocyanins were cyanidin-glycosides. Alkaline hydrolysis demonstrated that if these anthocyanins were acylated the acyl group was caffeic acid. Using the degradative techniques described before, it was possible to elucidate the structure of these anthocyanins as: cyanidin 3-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-rhamnosyl(1 → 6)glucoside, cyanidin 3-(4-caffeylrhamnosyl(1 → 6)glucoside), cyanidin 3-(rhamnosyl(1 → 6)glucoside)-5-glucoside, cyanidin 3-(4-caffeylrhamnosyl(1 → 6)glucoside)-5-glucoside and acylated cyanidin 3-glucoside and cyanidin 3,5-diglucoside.

Oxidation of acylated cyanidin 3,5-diglucoside with sodium periodate, followed by reduction with sodium borohydride and hydrolysis with hydrochloric acid yielded only glycerol. The acyl moiety has therefore to be bound at the 6-position of the 3-O-bound glucose (Watanabe *et al.* [14]). Alkaline hydrolysis liberated caffeic acid. A peak at 329 nm in the UV-spectrum also pointed to acylation with caffeic acid. NMR-data of the purified caffeylglucoside obtained by peroxide oxidation of the acylated diglucoside confirmed that the caffeyl group is bound at the 6-position of the 3-O-bound glucose. The following structure can be proposed for the acylated cyanidin-diglucoside: cyanidin 3-(6-caffeyl-β-D-glucoside)-5-β-D-glucoside.

The concentration of the acylated cyanidin 3-glucoside was to low to determine the position of the acyl moiety at the sugar molecule. From the UV-spectrum and alkaline hydrolysis it followed, however, that the acyl group of this compound probably also is caffeic acid.

(ii) Pink flowers. From petals of pink flowers of *p/p* plants with different *M*, *N* and *Ac* genotype combinations seven different orange-red anthocyanins were isolated. No bathochromic shift in the UV-spectra of these anthocyanins occurs in the presence of aluminium chloride. The aglycone obtained by total hydrolysis was identified by spectral analysis and co-chromatography in various solvents as pelargonidin (see experimental).

By means of the same degradation techniques described before the following anthocyanins were identified: pelargonidin 3-glucoside, pelargonidin 3,5-diglucoside, pelargonidin 3-rhamnosyl(1 → 6)glucoside, pelargonidin 3-(rhamnosyl(1→ 6)glucoside)-5-glucoside and acylated pelargonidin 3,5-diglucoside, pelargonidin 3-rhamnosyl(1 → 6)glucoside and acylated pelargonidin 3-rhamnosyl(1 → 6)glucoside)-5-glucoside.

Only one acylated pelargonidin-glycoside was found in amounts big enough to elucidate its structure. From the spectrum it could be concluded that this anthocyanin (pg-tri-ac) was acylated with only one acyl group ($E_{313}/E_{517} = 0.46$). The acyl moiety, obtained by alkaline hydrolysis, was chromatographically and spectroscopically indistinghuishable from an authentic sample of *p*-coumaric acid. Both the acyl group and *p*-coumaric acid were visible as blue spots after spraying with *p*-nitroanaline and as orange-red spots when sprayed with diazotized sulfanilic acid (Krebs *et al.* [12]). The NMR-spectra of both compounds were identical.

Partial hydrolysis yielded pelargonidin, pelargonidin 3-glucoside, pelargonidin 3-rhamnosyl(1 → 6)glucoside and pelargonidin 3-(rhamnosyl(1 → 6)glucoside)-5-glucoside. Total hydrolysis yielded the aglycone pelargonidin and the sugars rhamnose and glucose in a molar ratio of 1 : 1 : 2. Peroxide oxidation followed by alkaline hydrolysis liberated rutinose. From the E_{440}/E_{517} it followed that the 5-hydroxyl group was substituted.

Peroxide oxidation of pg-tri-ac produced a *p*-coumarylrutinoside. Partial hydrolysis of this compound yielded β-D-glucose and a *p*-coumarylrhamnoside.

Table II. Spectra and chromatographic data of the anthocyanins and their respective aglycone's isolated from petals of various genotypes of *S. dioica*.

Anthocyanin	HR_F-values				UV-absorption maxima in		
	paper			Si gel EFW	ethanol, 0.01% HCl [nm]	+$AlCl_3$ [nm]	$E_{440}/E_{vis\ max}$ [×100]
	BuHCl	BAW	HCl				
a. cyanidin-glycosides							
m	23	37	7	50	277, 538	+30	25
m-ac	34	47	20	(34)	276, 330, 538	+36	26
d	7	26	16	14	279, 535	+33	12
d-ac	16	30	27	11	279, 329, 535	+34	13
rhgl	24	33	15	46	277, 539	+33	26
rhgl-ac	46	47	31	31	277, 332, 538	+35	23
tri	9	22	35	16	278, 535	+36	13
tri-ac	14	32	48	11	279, 329, 535	+36	13
aglycone	69	69	2	96	277, 546	+18	25
b. pelargonidin-glycosides							
pg-m	41	43	11	62	272, 517	0	38
pg-d	14	30	19	22	270, 514	0	18
pg-d-ac	25	36	31	19	— —	—	—
pg-rhgl	35	46	20	66	271, 518	0	39
pg-rhgl-ac	66	57	34	44	— —	—	—
pg-tri	18	30	40	20	272, 518	0	19
pg-tri-ac	27	37	49	10	271, 313, 517	0	21
aglycone	80	82	2	97	270, 530	0	38

m = cyanidin 3-glucoside; m-ac = cyanidin 3-(6-caffeylglucoside) ?; d = cyanidin 3,5-diglucoside; d-ac = cyanidin 3-(6-caffeylglucoside)-5-glucoside; rhgl = cyanidin 3-rhamnosylglucoside; rhgl-ac = cyanidin 3-(4-caffeylrhamnosylglucoside); tri = cyanidin 3-rhamnosylglucoside-5-glucoside; tri-ac = cyanidin 3-(4-caffeylrhamnosylglucoside)-5-glucoside; pg-m = pelargonidin 3-glucoside; pg-d = pelargonidin 3,5-diglucoside; pg-d-ac = acylated pelargonidin 3,5-diglucoside; pg-rhgl = pelargonidin 3-rhamnosylglucoside; pg-rhgl-ac = acylated pelargonidin 3-rhamnosylglucoside; pg-tri = pelargonidin 3-rhamnosylglucoside-5-glucoside; pg-tri-ac = pelargonidin 3-(4-*p*-coumarylrhamnosylglucoside)-5-glucoside.

Oxidation of the purified anthocyanin pg-tri-ac with sodium periodate followed by reduction with sodium borohydride and hydrolysis with hydrochloric acid yielded both glycerol and 1,2,3-butanetriol which by analogous reasoning as with compound tri-ac, demonstrates that the acyl group is bound at the 4-position of the rhamnose molecule. This was confirmed by NMR-spectroscopy. The acylated orange-red anthocyanin present in petals of *p/p* plants therefore is: pelargonidin 3-(4-*p*-coumaryl-α-L-rhamnosyl(1 → 6)-β-D-glucoside)-5-β-D-glucoside.

The different anthocyanins found in various genotypes are presented in Table III. These results show that the structure of anthocyanins in petals of *S. dioica* is governed by the genes *P*, *M*, *N* and *Ac*.

Gene *Ac* controls the acylation of the anthocyanins. Depending on the allele present of gene *P* either *p*-coumaric acid (in *p/p* plants) or caffeic acid (in *P/P* plants) is bound at the 4-position of the rhamnose molecule bound to the 3-O-glucose. Gene *P* seems to control both the introduction of a hydroxyl group at the 3′-position of the anthocyanidin skeleton, *i.e.* the conversion of pelargonidin into cyanidin and the conversion of the acyl group *p*-coumaric acid into caffeic acid (Kamsteeg *et al.* [17]). Gene *M* governs the glucosylation of both cyanidin and pelargonidin 3-glucosides to their respective 3,5-diglucosides, whereas gene *N* governs the attachment of rhamnose to the glucose at the 3-position of the anthocyanidin skeleton of both the 3-glucosides and 3,5-diglucosides of pelargonidin and cyanidin.

The acylation controlled by gene *Ac* is quite complete when glucose(6 → 1)rhamnose is bound at the 3-position of the anthocyanidin skeleton. When only glucose is bound at the 3-position, however, only a minute part of the pigment is acylated. Moreover, in this case the acylation doesn't take place at the 4-position, but at the 6-position of the glucose molecule.

Our biochemical studies on genetic control of anthocyanin biosynthesis in *Silene* demonstrated that gene *M* controls the transfer of glucose from UDP-glucose to the 5-hydroxyl group of anthocyanidin 3-rhamnosyl-glucosides and gene N controls the transfer of rhamnose from UDP-rhamnose to the 3-O-bound glucose of cyanidin and pelargonidin 3-glucosides. Although no gene responsible for the anthocyanidin 3-glucosylation could be detected – no plants with solely an anthocyanidin aglycone in the petals have been found – the enzymatic formation of cyanidin and pelargonidin 3-glucoside from UDP-glucose and cyanidin-, respectively pelargoni-

Table III. Anthocyanins present in petals of various genotypes of *S. dioica*.

Genotype	Anthocyanins present														
	Cyanidin-glycosides								Pelargonidin-glycosides						
	m	m-ac	d	d-ac	rhgl	rhgl-ac	tri	tri-ac	pg-m	pg-d	pg-d-ac	pg-rhgl	pg-rhgl-ac	pg-tri	pg-tri-ac
P. mm nn ac ac	10														
P. mm nn Ac.	10	×													
P. M. nn ac ac	5		5												
P. M. nn Ac.	4		4	2											
P. mm N. ac ac					10										
P. mm N. Ac.					×	10									
P. M. N. ac ac							10								
P. M. N. Ac.							×	10							
pp mm nn ac ac									10						
pp M. nn ac ac									5	5					
pp M. nn Ac.									4	4	2				
pp mm N. ac ac												10			
pp mm N. Ac.												×	10		
pp M. N. ac ac														10	
pp M. N. Ac.														×	10

The numbers give the relative amounts of different anthocyanins present, to the total amount of anthocyanin, which is tentatively assigned 10 for all genotypes. "×" only a trace is present. For abbreviations see Table II.

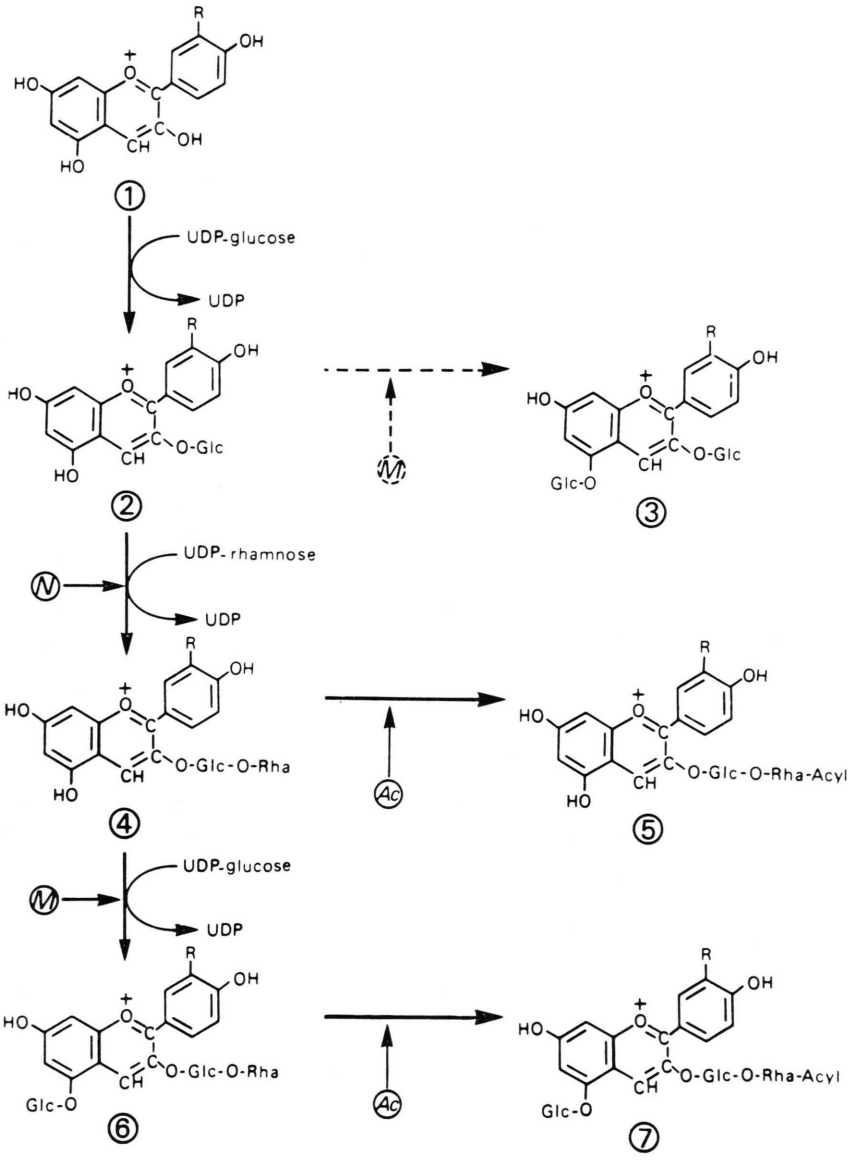

Fig. 1
Tentative biosynthetic pathway for anthocyanin formation in *S. dioica* (in *p/p* plants pelargonidin, R=H; in *P/P* plants cyanidin, R=OH). 1. aglycone, 2. anthocyanidin 3-glucoside, 3. anthocyanidin 3,5-diglucoside, 4. anthocyanidin 3-rhamnosylglucoside, 5. anthocyanidin 3-(4-acylrhamnosylglucoside), 6. anthocyanidin 3-rhamnosylglucoside-5-glucoside, 7. anthocyanidin 3-(4-acylrhamnosylglucoside)-5-glucoside.

din chloride has been demonstrated. The elucidation of the biosynthetic steps controlled by the other genes *P* and *Ac* is in progress.

From the results presented in this paper and our results from biochemical genetical studies (Kamsteeg *et al.* [18, 19]) on anthocyanin biosynthesis the following tentative biosynthetic pathways can be derived for anthocyanin biosynthesis in *S. dioica* (Fig. 1).

Experimental

Plant material: Silene dioica was grown in the open in the experimental garden of the Department of Population and Evolutionary Biology of the University of Utrecht. Growing conditions and crossing methods were the same as published before (Nigtevecht [20]). The petals were collected and extracted in 0.1% acetic acid in 70% methanol. Until use, the extract was stored under nitrogen at $-18\,^{\circ}C$.

Isolation of the anthocyanins: The methanol extract was homogenated with an Ultra-Turrax at 20 000 rpm, and centrifuged at $40\,000 \times g$. The supernatant was filtered and concentrated under reduced pressure. The concentrated filtrate was applied as a band on Whatman III paper and developed in the solvent system (*BAW*) *n*-butanol-acetic acid-

water (4 : 1 : 5, upper phase). The anthocyanin band was cut into pieces, homogenated with an Ultra-Turrax in 70% methanol, 0.1% hydrochloric acid and centrifuged. The concentrated supernatant was then applied as a band on Whatman III paper and developed in 1% hydrochloric acid, and eluted in ethanol, 0.01% hydrochloric acid.

Sugar identification: The anthocyanin to be hydrolyzed was suspended in 1 ml 2 N hydrochloric acid and hydrolyzed for one hour in a fused tube in a boiling waterbath. The aglycone formed was extracted with isoamyl alcohol and concentrated to dryness. The sugar containing waterphase was neutralized over a small column (1 × 5 cm) of a mixed bed ionexchanger (Merck V) and concentrated under reduced pressure. The released sugars were identified by means of co-chromatography with appropiate sugar references in the following combinations of thin layers and solvent systems (Lewis *et al.* [21]): (i) Si gel G buffered with 20 mM sodium acetate: propanon-2-butanone-tertiary butanol-water (15 : 30 : 40 : 15, v/v/v/v), (ii) kieselguhr buffered with 20 mM sodium acetate: ethyl acetate-isopropanol-water (65 : 24 : 12, v/v/v) and cellulose MN 300, developed twice with formic acid-2-butanone-tertiary butanol-water (15 : 30 : 40 : 15, v/v/v/v). The sugars were visualized by spraying with a mixture of 2.58 g aniline hydrogenphtalate in 100 ml water-saturated *n*-butanol, followed by heating for 5 minutes at 110 °C (Krebs *et al.* [12]).

Aglycone identification: The aglycone extracted was identified by co-chromatography with references on Whatman I paper in the solvent systems: isopropanol-2 N hydrochlorid acid (1 : 1, v/v), (cyanidin. R_F 0.38; pelargonidin 0.56) and forestal (acetic acid-hydrochloric acid-water (30 : 3 : 10, v/v/v)), (R_F cyanidin 0.49; pelargonidin 0.66). UV-spectra were recorded in ethanol, 0.01% hydrochloric acid. Cyanidin, λ_{max} in nm, 277, 325^{sh}, 382^{sh}, 455^{sh} and 546. Pelargonidin, 270, 329^{sh}, 435 and 530. IR-spectra were recorded in potassium bromide pellets. Cyanidin λ_{max} in cm^{-1}: 1442, 1515, 1570, 1610, 1637. Pelargonidin, 1443, 1508, 1526, 1575, 1605, 1638.

Alkaline hydrolysis of acylated anthocyanins: The purified compound (10 mg) was hydrolysed in the dark under nitrogen atmosphere in 1 ml 2 N potassium hydroxyde for 30 minutes at 0 °C. The solution was then acidified with 3 N hydrochloric acid up to pH 5 and concentrated under reduced pressure. The acyl compound was extracted with 5 ml diethylether and after concentration purified on TLC plates: Si gel GF 254 and Si gel G in the solvent system benzene-*p*-dioxane-acetic acid (90 : 25 : 4, v/v/v), (R_F caffeic acid 0.20, *p*-coumaric acid 0.39) and benzene-methanol-acetic acid (45 : 8 : 4, v/v/v) (Patuska [22]), (R_F caffeic acid 0.43, *p*-coumaric acid 0.50). The identification of the acyl compound was done by co-chromatography with various references. The hydroxycinnamic acids were visible on Si gel GF 254 plates under short wave UV light and on Si gel G plates after spraying either with 2% aluminium chloride, 3% iron (III) chloride, *p*-nitroaniline or sulfanilic acid (Krebs *et al.* [12]). UV-spectra were recorded in ethanol. Acyl group of cyanidin-glycosides, λ_{max} in nm, 230, 285 and 315. Acyl group of pelargonidin-glycosides 223, 290 and 311. NMR-spectra were recorded with a Varian A/60 MHz. Acyl group of cyanidin-glycosides: (60 MHz, DMSO d_6, δ TMS = 0.00), vinylic protons 6.30 (1 H, d, J = 16 Hz) and 7.60 (1 H, d, J = 16 Hz), aromatic protons 6.80 – 7.20 (3 H, m) and hydroxyl protons 9.82 (3 H, bs). Acyl group of pelargonidin-glycosides: (60 MHz, DMSO d_6, δ TMS = 0.00), vinylic protons 6.28 (1 H, d, J = 12.8 Hz) aromatic and vinylic protons 6.88 (3 H, m) and 7.58 (2 H, m) and hydroxyl protons 10.18 (2 H, bs).

Determination of molar ratio's: (i) molar ratio sugar/aglycone. The acylated anthocyanins suspended in ethanol, 5 N hydrochloric acid were refluxed for 20 minutes under nitrogen in the dark. The solution was cooled and diluted with ethanol to 100 ml and the aglycone concentration was determined by measuring the absorption, cyanidin 547 nm (ε 34 700, Ribéreau-Gayon [23]); pelargonidin 530 nm (ε 32 000, Weast [24]). The molar quantity of sugars in the hydrolysate was assayed according to Morris [25].

(ii) The molar ratio rhamnose/glucose was determined according to the method of Pridham [9].

(iii) The molar ratio anthocyanin/acyl moiety was determined according to Harborne [4] ($E_{300-330}/E_{vis.max.}$).

Oxidation with hydrogen peroxide: The purified anthocyanin (10 mg) was dissolved in 2 ml methanol and oxidized with 0.4 ml 30% hydrogen peroxide for 4 hours in the dark (Chandler and Harper [11]). A few grains of palladium-charcoal catalyst were added to effect decomposition of excess per-

oxide. After 20 hours in the dark, 0.1 N ammonia was added to pH 8.0 and the solution heated for 1 – 5 minutes under nitrogen in a boiling water-bath. The sugars or sugar esters were purified by two-dimensional paper chromatography on Whatman III paper in the solvent systems *n*-butanol-acetic acid-water (4 : 1 : 5, v/v/v, upper phase) and *n*-propanol-ethyl acetate-water (7 : 1 :2, v/v/v).

Partial and total hydrolysis: (i) Procedure of Lynn and Luh [26]. About 1 mg anthocyanin was hydrolysed in 2 ml of 1 N hydrochloric acid at 97 °C using the method of Lynn and Luh. Aliquots were withdrawn at 5 minutes intervals, spotted on Si gel G plates and developed in ethyl acetate-formic acid-water (68 : 14 : 18, v/v/v). (ii) Hydrolysis with Dowex 50 W – X 8. The purified acylated anthocyanin was absorbed on Dowex 50 W – X 8 (H^+-form) which was previously washed with water and methanol, and heated for 30 minutes at 80 °C under continuous stirring. The ion-exchanger was washed two times with water and the combined filtrates were concentrated under reduced pressure (Watanabe *et al.* [14]). The sugar or sugar esters released were purified as described above. (iii) Hydrolysis with acetic acid. The purified acylsugars, obtained by hydrogen peroxide oxidation of the acyl-anthocyanin were hydrolysed with 5% acetic acid for 5 – 20 minutes at 80 °C.

Oxidation with sodium metaperiodate: 20 mg of the acylated anthocyanin and 200 mg sodium periodate were dissolved in 50 ml water and kept in the dark for 17 hours. A solution of 1.5 g sodium borohydride in 10 ml water was added dropwise. After 6 hours in the dark the excess sodium borohydride was decomposed by adding 2 N hydrochloric acid to pH 2.0. The solution was desalted over a mixed bed ionexchanger (Merck V), concentrated under reduced pressure and hydrolysed with 2 N hydrochloric acid for 30 minutes at 100 °C. After desalting, the hydrolysate was chromatographed on Whatman I paper in the solvent systems *n*-butanol-acetic acid-water (4 : 1 : 5, v/v/v, upper phase) and ethyl acetate-pyridine-water (7 : 2 : 1, v/v/v), R_F glycerol 0.36, 1,2,3-butanetriol 0.55. The polyalcohols were visible after spraying with potassium metaperiodate, followed by spraying with a borax-starch solution (Metzenberg [15]).

NMR-spectral data: NMR spectral data of the acylglycosides isolated after peroxide oxidation of acyl-anthocyanins. The spectra were recorded on a Varian 60 MHz. Hydrogen peroxide oxidation of anthocyanin: pelargonidin 3-(4-*p*-coumaryl-α-L-rhamnosyl(1 → 6)-β-D-glucoside)-5-β-D-glucoside yielded 4-*p*-coumaryl-α-L-rhamnosyl(1 → 6)-β-D-glucose: (60 MHz, DMSO d_6, δ TMS = 0.00 – 0.30), aromatic protons coumaryl ester 5.80 – 7.70 (2 H, d, J = 8.5 Hz), vinylic protons 6.20 and 7.50, (2 H, m, J = 16 Hz), methyl protons of rhamnose 1.68 (3 H, q), rhamnose protons 3.60 – 5.30 (5 H, m), glucose protons 2.90 – 5.80 (7 H, m). H – C_{acyl} 4.46 (2 H, d, J = 3 Hz), coupling constant 5.09 (2 H, t, J [between proton H – C_{acyl} and its adjacent proton] = 9 Hz). Hydrogen peroxide oxidation of anthocyanin cyanidin 3-(4-caffeyl-α-L-rhamnosyl(1 → 6)-β-D-glucoside)-5-β-D-glucoside yielded 4-caffeyl-α-L-rhamnosyl(1 → 6)-β-D-glucose: (60 MHz, DMSO d_6, δ TMS = 0.00 – 0.40), aromatic protons 5.80 – 7.80 (3 H, t, J = 8.5 Hz), vinylic protons 7.00 – 7.40 (2 H, m, J = 16 Hz), methyl protons of rhamnose 1.70 (3 H, q), rhamnose protons 3.60 – 5.30 (5 H, m), glucose protons 2.90 – 5.80 (7 H, m). H – C_{acyl} 4.46 (2 H, d, J = 3 Hz), coupling constant 5.09 (2 H, t, J = 9 Hz). Hydrogen peroxide oxidation of anthocyanin cyanidin 3-(6-caffeyl-β-D-glucoside)-5-β-D-glucoside yielded 6-caffeyl-β-D-glucose: (60 MHz, DMSO d_6, δ TMS = 0.00 – 0.40), vinylic protons 7.50 (1 H, d, J = 16 Hz) and 6.20 (1 H, d, J = 16 Hz), aromatic protons 6.80 (2 H, d, J = 8.5 Hz) and 7.40 (2 H, d, J = 8.5 Hz), glucose protons 2.90 – 5.80 (7 H, m).

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