Phytochemistry, 1976, Vol. 15. pp 1917–1718. Pergamon Press. Printed in England.

PLEIOTROPIC EFFECT OF A PELARGONIDIN-HYDROXYLATION GENE IN *SILENE DIOICA*?

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(*Revised received* 30 *June* 1976)

Key Word Index—*Silene dioica*; Caryophyllaceae; red campion; cyanidin; pelargonidin; isovitexin glycosides; hydroxylation; biosynthesis; pleiotropic effect.

Abstract—In petals of *Silene dioica* a gene *P* has been identified, which controls the 3'-hydroxylation of the B-ring of pelargonidin to cyanidin. Another gene *Ac* controls the acylation of the terminal sugar at the 3-position of anthocyanin 3-rhamnosylglucoside-5-glucosides. In *p/p* plants the bound acyl group is *p*-coumaric acid; in *P/P* plants, however, it is caffeic acid. Gene *P* seems to exert a pleiotropic effect: it not only controls the hydroxylation of the B-ring of pelargonidin but also that of the acyl group.

INTRODUCTION

From the results of studies on flavonoid biosynthesis in *Petunia hybrida*, Hess [1,2] proposed the so-called "cinnamic acid starter hypothesis". According to this hypothesis, the hydroxy and methoxy substitution pattern of the B-ring of flavonoids is determined at the cinnamic acid level. Supporting evidence for this hypothesis was supplied by Meier and Zenk [3] and Steiner [4], who showed that labelled cinnamic acids were incorporated into the correspondingly substituted anthocyanins.

Modifications in the substitution pattern of the B-ring can also occur at the chalcone/flavanone, or even later stages of the flavonoid biosynthetic pathway [5–9]. Methyltransferases [10,11], phenolases [13–15] and hydroxylases [8] capable of modifying the B-ring of flavonoids have been demonstrated. However, because of the broad substrate specificity of some of these enzymes, especially the phenolases, additional criteria are needed to demonstrate that these enzymes also exert the same function *in vivo*.

In this paper we discuss a gene *P* in *S. dioica* which in *P/.Ac/.* plants not only hydroxylates pelargonidin to cyanidin, but is apparently also able to hydroxylate the acyl group bound to the cyanidin-glycoside to caffeic acid.

RESULTS AND DISCUSSION

In petals of *Silene dioica* cyanidin 3-rhamnosyl(1→6)-glucoside-5-glucoside and its acylated form, cyanidin 3-(4-caffeylrhamnosyl(1→6)glucoside)-5-glucoside are normally present. However, in a few plants, pelargonidin glycosides have been found. Crossing experiments (Table 1) demonstrate that gene *P* controls the introduction of the hydroxyl group at the 3'-position of the anthocyanidin skeleton and that another gene *Ac* controls the acylation of anthocyanins in petals of *S. dioica*. In petals of different genotypes the following substances are found: in *p/p ac/ac* plants pelargonidin 3-rhamnosyl(1→6)-glucoside-5-glucoside; in *p/p Ac/.* plants a mixture of pelargonidin 3-rhamnosyl(1→6)glucoside-5-glucoside and pelargonidin 3-(4-*p*-coumaryl(1→6)glucoside)-5-glucoside; in *P/. ac/ac* plants cyanidin 3-rhamnosyl(1→6)-glucoside-5-glucoside; in *P/. Ac/.* plants a mixture of cyanidin 3-rhamnosyl(1→6)glucoside-5-glucoside and cyanidin 3-(4-caffeylrhamnosyl(1→6)glucoside)-5-glucoside. In the presence of the dominant allele of *Ac* the cyanidin-glycoside present in *P* plants is acylated with caffeic acid, whereas in *p/p* plants the pelargonidin glycoside present is acylated with *p*-coumaric acid. Therefore the oxidation pattern of both the B-ring of the anthocyanidin molecule and of the acyl group bound to the terminal sugar at the 3-position of the anthocyanin-glycoside appears to be controlled by gene *P*. Gene *P* thus seems to exert a pleiotropic effect: it not only controls the hydroxylation of the anthocyanidin molecule, but also that of the acyl moiety. An alternative explanation is that gene *P* controls the hydroxylation of *p*-coumaric acid to caffeic acid, which then is used both as a precursor in the anthocyanin biosynthesis and in the acylation step. In this case, there is no pleiotropy.

Our genetical results contrast with the recent findings of Fritsch and Grisebach [8] in *Haplopappus gracilis*, who demonstrated a microsomal hydroxylase which converted naringenin to eriodictyol and dihydrokaempferol. This hydroxylase however, was unable to convert *p*-coumaric acid to caffeic acid.

To elucidate this discrepancy between our results on *S. dioica* and the work of Fritsch and Grisebach on *H. gracilis*, biochemical studies have been started to identify the protein controlled by gene *P*. This gene *P* is not involved in flavone biosynthesis. Both in petals of *P/P* and *p/p* plants derivatives of isovitexin [16] are present. The possibility that anthocyanin- and flavone-glycoside biosynthetic pathways are separate cannot therefore be excluded. There is some evidence that multienzyme systems are involved in flavonoid biosynthesis. [17]

EXPERIMENTAL

Plant material. Silene was grown in the open in the experimental garden of the Department of Population and Evolutionary Biology of Utrecht University. Growing conditions and crossing methods were the same as published before. [18] The petals were collected and extracted with 1% HCl in 70% MeOH. Until use, the extract was stored under N_2 at −18°.

Isolation of the anthocyanins. MeOH extract was homogenated at 20000 rpm. Homogenate was centrifuged for

Table 1. Genetic control of pelargonidin hydroxylation and anthocyanin acylation in petals of *S. dioica*

No	Type of cross		*N*	anthocyanin offspring observed	expected	Chi²	*P*
1.	cy (*P/P*)	× pg (*p/p*)	102	cy 102 (*P/p*)	102	—	—
2.	(cy × pg) (*P/p*)	× pg (*p/p*)	160	cy 86 pg 74 (*P/p*) (*p/p*)	80:80	0.9	>0.30
3.	cy (*P/P ac/ac*)	× cy-ac (*P/P Ac/Ac*)	179	cy-ac 179 (*P/P Ac/ac*)	179	—	—
4.	(cy × cy-ac) (*P/P Ac/ac*)	× (cy × cy-ac) (*P/P Ac/ac*)	89	cy-ac 65 cy 24 (*P/P Ac/.*) (*P/P ac/ac*)	66¾:22¼	0.18	>0.50
5.	pg (*p/p ac/ac*)	× pg-ac (*p/p Ac/Ac*)	69	pg-ac 69 (*p/p Ac/ac*)	69	—	—
6.	(pg × pg-ac) (*p/p Ac/ac*)	× (pg × pg-ac) (*p/p Ac/ac*)	73	pg-ac 52 pg 21 (*p/p Ac/.*) (*p/p ac/ac*)	54¾:18¼	0.54	>0.30
7.	(cy × pg-ac) (*P/p Ac/ac*)	× pg (*p/p ac/ac*)	284	cy 73 cy-ac 77 pg 62 pg-ac 72 (*P/p ac/ac*) (*P/p Ac/ac*) (*p/p ac/ac*) (*p/p Ac/ac*)	71:71:71:71	1.72	>0.50
8.	(cy × pg) (*P/p ac/ac*)	× (pg × pg-ac) (*p/p Ac/ac*)	104	cy 28 cy-ac 31 pg 19 pg-ac 26 (*P/p ac/ac*) (*P/p Ac/ac*) (*p/p ac/ac*) (*p/p Ac/ac*)	26:26:26:26	2.99	>0.30

The genotype of the parents and the expected genotype of the offspring is given within brackets. Petals of cy-plants (*P/p ac/ac* and *P/P ac/ac*) contained cyanidin 3-rhamnosylglucoside-5-glucoside; cy-ac-plants (*P/P Ac/Ac* and *P/. Ac/.*), cyanidin 3-rhamnosylglucoside-5-glucoside and cyanidin 3-(4-caffeylrhamnosylglucoside)-5-glucoside; pg-plants (*p/p ac/ac*), pelargonidin 3-rhamnosylglucoside-5-glucoside and pg-ac-plants (*p/p Ac/Ac* and *p/p Ac/ac*), pelargonidin 3-rhamnosylglucoside-5-glucoside and pelargonidin 3-(4-*p*-coumarylrhamnosylglucoside)-5-glucoside.

30 min at 40000 *g*. Supernatant was filtered and concentrated at red pres. Concentrated filtrate was applied as a band on Whatman III paper and developed in *n*-BuOH–HOAc–H_2O (4:1:5, upper phase). Anthocyanins were eluted in 70% MeOH, 0.1% HCl. Eluate was conc at red pres. Concentrated eluate was then applied as a band on Whatman III and developed in 1% HCl, and again eluted in EtOH, 0.01% HCl.

Alkaline hydrolysis of acylated anthocyanins. The purified compound (10 mg) was in the dark under N_2 hydrolysed in 1 ml 10% KOH for 30 min at 0°. The soln was then acidified with 3N HCl up to pH 5 and conc at red pres. The acyl compound was extracted with 5 ml Et_2O and after concn purified on TLC plates: Si gel GF 254 and Si gel G in benzene–dioxane–HOAc (90:25:4) and benzene–MeOH–HOAc (45:8:4) respectively [19]. 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% $AlCl_3$, 3% $FeCl_3$, *p*-nitroaniline or sulfanilic acid [20].

Spectral data. UV-spectra were recorded in EtOH, 0.01% HCl. Cyanidin 3-(4-caffeylrhamnosyl(1→6)glucoside)-5-glucoside, λ_{max} in nm: 279, 329 and 535; *A*329/535 = 0.46 [21]. Cyanidin 3-rhamnosyl(1→6)glucoside-5-glucoside: 278 and 535 nm. Pelargonidin 3-(4-*p*-coumarylrhamnosyl(1→6)glucoside-5-glucoside: 271, 313 and 517 nm; *A*313/517 = 0.46. Pelargonidin 3-rhamnosyl(1→6')glucoside-5-glucoside: 272 and 518 nm. Spectra of acyl compounds were recorded in EtOH. Acyl group of cyanidin-glycoside: 230, 285 and 315 nm. Acyl group of pelargonidin-glycoside: 223, 290 and 311 nm. NMR spectra were recorded on a Varian 60 MHz. Acyl group of cyanidin-glycoside: (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-glycoside: (60 MHz, DMSO d_6, δ TMS = 0.00) vinylic proton 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*).

Acknowledgements—We are much indebted to Dr. F. J. E. M. Küppers of the Laboratory of Organic Chemistry, Utrecht, who recorded and interpreted the NMR spectra. We also thank Mrs. A. Oostra for her skilfull assistance.

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