Phytochemistry, 1972, Vol. 11, pp. 1083 to 1088. Pergamon Press. Printed in England.

BIOCHEMICAL GENETICS OF PIGMENTATION IN *PISUM SATIVUM*

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(*Received* 5 *August* 1971)

Abstract—The anthocyanins and flavones present in different colour forms of *Pisum sativum* have been identified and related to the genetic constitution of the plants. Two new anthocyanin pigments, delphinidin 3-sophoroside-5-glucoside, and delphinidin 3-sambubioside-5-glucoside have been isolated from purple pods and from flowers with the *cr cr* genotype. Chemical effects have been designated to the *B*-(hydroxylation) and *Cr*-(methylation) loci, whereas *Am*, *Ar* and *Ce* appear to have only quantitative effects. Variation of genetic expression in different tissues of the plant is demonstrated.

INTRODUCTION

INVESTIGATIONS into the inheritance of flower colour in *Pisum* began with the classical studies of Mendel,[1] who crossed purple-flowered with white-flowered plants. After the re-discovery of Mendel's work in 1901, the genetic difference which he observed was ascribed to the locus *A*. Subsequent genetical investigations have demonstrated the involvement of six major loci in the production of flower colour in peas: *A*: Mendel,[1] Tschermak;[2] *B*: Tschermak;[2] *Ar*: Tedin;[3] *Cr*: de Haan,[4] Fedotov;[5] *Am*: de Haan;[4] and *Ce*: Wellensiek.[6] Other gene systems are known which determine pigmentation elsewhere in the plant.[7,8] These include the loci *Gp*, *Pu* and *Pur*, which are concerned with pod colouration. Superimposed on these 'pigment production' genes, are the effects of other genes concerned with distribution and patterning of the pigments.

In a preliminary investigation using only a limited number of colour forms, Dodds and Harborne[9] identified a range of anthocyanins in flowers with genotypes differing at the *b* and *cr* loci, and were able to propose a chemical basis for the expression of these genes. The major anthocyanins present in red-coloured pods of plants with the genotype *A b Cr* were also identified. In this investigation we have extended the earlier work to cover a wider range of genotypes, and have also examined in some detail the pigmentation of organs other than flowers.

[1] G. MENDEL, *Verh. des Naturf. Vereines in Brunn*, **4**, 3 (1865).
[2] E. VON TSCHERMAK, *Zeits. Ind. Abst. Vererb.* **7**, 81 (1912).
[3] H. TEDIN, *Hereditas* **1**, 68 (1920).
[4] H. DE HAAN, *Genetica* **12**, 321 (1930).
[5] V. S. FEDOTOV, *Proc. USSR Congr. Genetics* **2**, 523 (1930).
[6] S. J. WELLENSIEK, *Genetica* **25**, 525 (1951).
[7] S. BLIXT, *Agri. Hort. Genet.* **20**, 95 (1962).
[8] H. LAMPRECHT, *Agri. Hort. Genet.* **21**, 19 (1963).
[9] K. S. DODDS and J. B. HARBORNE, *A. Rep. John Innes Inst.* **34** (1963).

RESULTS

Identification of Delphinidin 3-sophoroside-5-glucoside, and Delphinidin 3-sambubioside-5-glucoside

These two anthocyanins are new plant pigments. The glycosides were first identified as major constituents of purple pods of *Pisum*, and were later found to be minor constituents of crimson (*cr cr*) and purple (wild type) flowers. The related cyanidin glycosides had earlier been reported as constituents of red pods in *Pisum*.[9]

The structure of the disaccharides substituted at the 3 positions were not determined unambiguously. However, the intermediates from partial hydrolyses had identical chromatographic behaviour to reference samples of delphinidin 3-sophoroside and delphinidin 3-sambubioside. Delphinidin 3-sophoroside-5-glucoside and delphinidin 3-sambubioside-5-glucoside have very similar mobilities in most of the usual chromatographic solvents. However, they are adequately separated using 3% HCl. The relevant chromatographic data are summarized in Table 1.

TABLE 1. R_f DATA OF NEW DELPHINIDIN ANTHOCYANINS

Anthocyanin	Solvent			
	BAW	5% HOAc	WAH	3% HCl
Dp 3GG, 5G	0·12	0·60	0·68	0·63
Dp 3GX, 5G	0·12	0·60	0·62	0·57

Anthocyanins Identified in the Various Genotypes of Pisum

These are listed in Table 2. Flowers of genotypes marked with an asterisk contained very little anthocyanin in relation to flowers of other genotypes. From the results in the table, it is clear that the loci *Am*, *Ar* and *Ce* have quantitative effects only. They do not affect the chemistry of the anthocyanins.

Other Pigments

Of the thirty-two different cyanic lines of *Pisum* available for study, all but two, S1228 and S1539, contained flavones in the flowers. Five compounds, all *C*-glycosides, were isolated and four were identified (Table 2). The fifth, a very minor constituent, is reported as an apigenin 6-*C*-glycoside though its sugar moiety remains unidentified.

The complement of *C*-glycosyl-flavone was invariant, and all the lines investigated contained the same pigments. However, the distribution of flavones differed from that of the anthocyanins, the former being found only in the standard petals, while the anthocyanins were found in both wings and standards. No flavonoid pigmentation was observed in the keel petals of the flowers. The qualitative survey of the two-dimenisonal chromatograms of flower petal extracts showed that flavones were present in highest concentrations in those flowers possessing little anthocyanin, that is, in genotypes containing recessive *ce* and *am*. The major leaf flavonoids are the same as those reported in the cultivated 'Alaska' pea, i.e. quercetin and kaempferol 3-triglucosides occurring as such and also acylated with *p*-coumaric acid. The sugar is a sophorose derivative, probably sophorotriose,[10] and thus the leaf flavonols are structurally related to some of the minor flower anthocyanins.

[10] J. B. HARBORNE, *Experientia* **19**, 7 (1963).

TABLE 2. FLAVONOID PIGMENTS IDENTIFIED IN *Pisum sativum*

Genotype	Anthocyanins
1. *Flowers*	
A Am Ar B Ce Cr *A *am* Ar B Ce Cr	Delphinidin, petunidin and malvidin 3-rhamnoside-5-glucosides and malvidin 3-rhamnoside. Minor pigments
A Am *ar* B Ce Cr A Am Ar B *ce* Cr	Delphinidin and petunidin 3,5-diglucoside, delphinidin 3-sophoroside-5-glucoside, 3-sambubioside-5-glucoside, and 3-sophoroside
A Am Ar *b* Ce Cr *A *am* Ar *b* Ce Cr *A *am ar b* Ce Cr A Am *ar b* Ce Cr A Am Ar *b ce* Cr	Pelargonidin, cyanidin and peonidin 3-rhamnoside-5-glucoside. Minor pigments Cyanidin 3-rhamnoside, 3-glucoside, 3,5-diglucoside, and 3-sambubioside-5-glucoside.
A Am Ar B Ce *cr* A Am *ar* B Ce *cr*	Delphinidin 3-glucoside, 3,5-diglucoside, 3-sophoroside-5-glucoside, and 3-sambubioside-5-glucoside. Minor pigments
*A Am Ar B *ce cr*	Delphinidin 3-sophoroside and 3-sambubioside
A Am Ar *b* Ce *cr* *A Am *ar b* Ce *cr*	Cyanidin glycosides only
2. *Pods*	
A Am Ar B Ce Cr Pur Pu Gp	Delphinidin and cyanidin 3-sophoroside-5-glucoside and 3-sambubioside-5-glucoside
A Am Ar *b* Ce Cr Pur Pu Gp	Cyanidin 3-sophoroside-5-glucoside and 3-sambubioside-5-glucoside
3. *Axils*	
A Am Ar B Ce Cr A *am* Ar B Ce Cr A Am *ar* B Ce Cr A Am Ar B *ce* Cr (S1458) A Am Ar B Ce *cr*	Delphinidin and cyanidin glycosides
A Am Ar B *ce* Cr (S1227)	Delphinidin, malvidin, cyanidin and peonidin glycosides
A Am *ar* B Ce *cr*	Delphinidin, malvidin and cyanidin glycosides
A Am Ar *b* Ce Cr A Am Ar *b* Ce *cr* A *am* Ar *b* Ce Cr	Cyanidin and peonidin glycosides
	Flavones
1. *Flowers* All genotypes with flavones present	Luteolin 6-*C*-glucoside (Iso-orientin) and 8-*C*-glucoside (Orientin). Apigenin 6-*C*-glucoside (Iso-vitexin), 8-*C*-glucoside (Vitexin) and 6-*C*-glycoside (Sugar unidentified)
2. *Leaves* All genotypes	Kaempferol and quercetin 3-sophorotriosides, as such and also acylated with *p*-coumaric acid

DISCUSSION

This survey has shown that, of the six major genes known to control flower colour in *Pisum*, *A* is necessary for general flavonoid production in the plant, and for anthocyanin production in the flowers, axils and pods; *B* and *Cr* modify the structure of the anthocyanins, but appear to have no effect on other classes of flavonoids, while the three other genes, *Am*, *Ar* and *Ce* have only quantitative effects. No flavonoids could be detected in eight lines with white flowers (genotype *a a*). Thus, in *Pisum*, as in *Antirrhinum*[11] and probably *Petunia*,[12] there are varieties which are totally lacking in flavonoids. It appears, therefore, that gene *A* in *Pisum* is necessary for production of both anthocyanins and flavones in the flowers.

The *B* locus in *Pisum* apparently controls the 5′-hydroxylation of the B-ring of the anthocyanins. No separate locus for the 3′-hydroxylation of anthocyanins, comparable to the *Sm* locus of *Lathyrus*, has been reported for *Pisum*, and none of the genotypes available for our study contained only pelargonidin, as would be expected if such a locus had been preserved in cultivation.

Genetic control of hydroxylation of anthocyanins has usually been found to be tissue specific. For example, in *Impatiens balsamina*,[13] the flowers may have delphinidin, cyanidin or pelargonidin, but the sepals of most genotypes have only cyanidin. Even when the genetic control of hydroxylation affects pigment synthesis throughout the plant, the expression of the genes may vary from one tissue to another. Thus, in *Primula sinensis*,[14] *K* types have delphinidin in both leaf and petal, while *k k* types have mainly cyanidin in the leaf, but mainly pelargonidin in the petals. In *Pisum*, gene *B* controls 5′-hydroxylation of the anthocyanins in the flowers, axils and pods. However, only in the flowers of *B* genotypes is the cyanidin of *b b* genotypes completely replaced by delphinidin. Axils and pods contain both delphinidin and cyanidin glycosides.

The locus *Cr* controls the methylation of anthocyanins in the flowers. Production of methylated glycosides in the axils is not related to the genetic constitution with respect to the *Cr* locus. Recessivity for *Cr*, while not allowing production of methylated anthocyanins in the flowers, does not necessarily prevent methylation of anthocyanins in the axils. No methylated anthocyanin has been found in pod material, although all lines under investigation were homozygous dominant at the *Cr* locus.

Also, it appears that plants of the genotype *cr cr* do not possess the rhamnosyl transferases which must be present in plants of genotype *Cr*. All flowers of the latter type possessed not only methylated anthocyanins, but also glycosides of the type 3-rhamnoside, and 3-rhamnoside-5-glucoside. The observation that methylation and glycosylation are under simultaneous control by a single locus has been noted in some other plants, for example, *Solanum*[15] and *Petunia*.[16] In these two instances, the gene concerned is thought to be tightly linked to the gene controlling acylation of anthocyanins, and the three different biochemical effects are thought to be part of a compound locus. However, in both *Solanum* and *Petunia*, the glycosylation reaction involved is the addition of glucose to the 5-position of the *A*-ring, while in *Pisum*, the glycosylation effect related to *cr cr* appears to be the lack of an enzyme required for the addition of rhamnose to position 3. Again in *Solanum*, methylation is

[11] T. A. GEISSMAN and J. B. HARBORNE, *Arch. Biochem. Biophys.* **55**, 477 (1955).
[12] G. MOSIG, *Z. Vererbungsl.* **91**, 171 (1960).
[13] R. E. ALSTON and C. W. HAGEN, *Genetics* **43**, 35 (1958).
[14] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 259, Academic Press, New York (1967).
[15] J. B. HARBORNE, *Biochem. J.* **74**, 262 (1960).
[16] C. MEYER, *Z. Vererbungsl.* **95**, 171 (1964).

complete in the flowers, but incomplete in the tubers, which suggests that the degree of methylation exhibits activity gradients. A similar situation occurs in *Pisum*, where delphinidin, petunidin and malvidin occur in *B* flowers, and pelargonidin, cyanidin and peonidin in *b b* flowers, but methylated anthocyanins may or may not occur in the axils of all genotypes, and have not been found in the pods.

Each of the *Pisum* genotypes investigated contained a complex mixture of anthocyanins, a situation observed before in *Lathyrus*[17] and *Streptocarpus*.[18] In fact, in both *Pisum* and *Lathyrus*, the major pigments of the flowers are the 3-rhamnoside-5-glucosides, with the minor pigments being a complex mixture of many different glycosidic types. The differences in the flower colour between the two genera are due to the fact that no specific methylating factor has been observed in *Lathyrus*, while in *Pisum* no specific 3′-hydroxylating locus has been recorded.

A single Mendelian gene not previously described in peas appears to determine flavone production in the flowers of *Pisum*, the presence of the dominant allele resulting in pigment production. This is similar to the report for *Petunia*.[12] Experimental data describing this new locus will be published elsewhere.

In contrast with an earlier report,[19] Harborne[17] found that related anthocyanins and flavonols occur together in the flowers of all varieties of *Lathyrus* that he examined. Thus in *Lathyrus*, the loci *E* and *Sm* which control hydroxylation of anthocyanins, also determine the flavonol hydroxylation pattern. In *Pisum*, the genes affecting flower colour operate only on the anthocyanins. The pattern of flavone *C*-glycosides was the same in all coloured flowers, irrespective of the flower colour genes. These genes also were without effect on the flavonols found in the vegetative tissues.

EXPERIMENTAL

Plant materials. Seeds from genetically pure lines were used throughout. The seeds were obtained from the Plant Breeding Institution, Weibullsholm, Landskrona, Sweden (S lines), and Dr. I. C. Murfet, Botany Department, University of Tasmania, Hobart (L lines), Table 3. Seeds were grown in a glasshouse in containers having 1 : 1 vermiculite–dolerite chips (¼in. mesh) solid medium. Plants were watered daily, and normal Hoagland's nutrient solution applied weekly.

Analysis and identification of pigments. Flowers were collected when petals were fully expanded and had developed maximum pigmentation. Wings, standards and keels were examined separately. The pods were collected after they had grown to full length, but before the seeds were fully developed. Pigmented axils were cut from the plant on the completion of flowering, but before the plants had dried.

Fresh tissue was extracted using MeOH : 1% HCl for anthocyanins, and 70% EtOH for other flavonoids and phenolics. The extracts were examined by two-dimensional chromatography on Whatman No. 1 chromatography paper using BAW (*n*-BuOH–HOAc–H_2O, 4 : 1 : 5, v/v, upper phase), and 5% HOAc. When it had been established that all lines of a particular genotype contained the same complement of pigments, most of the isolation work was carried out using extracts from a single line of that genotype. Anthocyanins, were isolated from the following lines:

A Am Ar B Ce Cr	L60	Purple flowers	Wings
A Am Ar *b* Ce Cr	S592	Pink flowers	Wings
A Am Ar B Ce *cr*	S1366	Crimson flowers	Wings
A Am Ar B Ce Cr Pur Pu Gp	S1017	Purple pods	
A Am Ar *b* Ce Cr Pur Pu Gp		Red pods	
Flavones were extracted from A *am* Ar B Ce Cr	S369	Pinkish-white flowers	Standards.

Purification of pigments was carried out by band-loading extracts onto Whatman 3MM chromatography paper, and developing in BAW, 5% HOAc, and for pod material, 3% HCl. The pigments were identified by

[17] J. B. HARBORNE, *Nature, Lond.* **187**, 240 (1960).
[18] W. J. C. LAWRENCE and V. C. STURGESS, *Heredity* **11**, 303 (1957).
[19] G. H. BEALE, G. M. ROBINSON, R. ROBINSON and R. SCOTT-MONCRIEFF, *J. Genet.* **37**, 375 (1939).

TABLE 3. *Pisum* VARIETIES USED FOR ANALYSIS AND IDENTIFICATION OF PIGMENTS

Genotype	Line No.	Colour
1. *Flowers*		
A Am Ar B Ce Cr	S577, S1017, S1402, S1516 L2, L41, L51, L60	Purple (Wild Type)
A *am* Ar B Ce Cr	S369, S1088, S1451	Pinkish-white
A *am* Ar *b* Ce Cr	S1512	
A *am ar b* Ce Cr	S1467	
A Am *ar* B Ce Cr	S25, S1391	Violet
A Am *ar b* Ce Cr	S1357	
A Am *ar* B Ce *cr*	S1325	
A Am *ar b* Ce *cr*	S1089	
A Am Ar *b* Ce Cr	S592, S1185, S1508, S1515, L13	Pink
A Am Ar *b ce* Cr	S1511	
A Am Ar *b* Ce *cr*	S1510, L12	
A Am Ar B *ce* Cr	S1227, S1458	Cerise
A Am Ar B *ce cr*	S1228, S1539	
A Am Ar B Ce *cr*	S1221, S1366	Crimson
aa———————	S102, S206, S680, S1143 S1241, S1525, L22, L23	White
2. *Pods*		
A Am Ar B Ce Cr Pur Pu Gp	S577, S1017, L41	Purple
A Am Ar *b* Ce Cr Pur Pu Gp	One line selected from the cross L41 × L13	Red

spectral and chromatographic analysis, comparison with authentic reference compounds, and by partial and complete hydrolysis, using the methods of Harborne[20,21] and Mabry *et al.*[22]

Acknowledgements—The authors are grateful to Dr. S. Blixt and Dr. I. C. Murfet who provided seeds of genetically pure lines, and to Dr. T. Mabry, Department of Botany, University of Texas, Austin, Texas, U.S.A., and Dr. B. H. Koeppen, Department of Food Science and Technology, University of Stellenbosch, Cape, South Africa, for gifts of reference flavones. That part of the investigation carried out in Tasmania was supported by the Australian Research Grants Committee.

[20] J. B. HARBORNE, *Biochem J.* **70**, 22 (1958).
[21] J. B. HARBORNE, *J. Chromatog.* **1**, 473 (1958).
[22] T. J. MABRY, K. R. MARKHAM and M. B. THOMAS, *The Systematic Identification of Flavonoids*, Springer-Verlag, Berlin (1970).

Key Word Index—Pisum sativum; Leguminosae; pea; flavonoids; anthocyanins; biochemical genetics.