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# ANTHOCYANIN BIOSYNTHESIS IN *PISUM*: SEQUENCE STUDIES IN PIGMENT PRODUCTION

CARMEL M. STATHAM and RONALD K. CROWDEN

Department of Botany, University of Tasmania, Box 252c, G.P.O., Hobart, Tasmania, Australia 7001

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**Key Word Index**—*Pisum*; *Lathyrus*; Leguminosae; anthocyanin; biosynthesis; sequence of accumulation; biochemical genetics; methylation; rhamnosylation.

**Abstract**—The sequence of anthocyanin accumulation during flower development in four flower-colour mutants of *Pisum* and in *Lathyrus odoratus* var. Chloe, shows a progression from methylated to unmethylated anthocyanidins, and the replacement of 3-*O*-rhamnoside by 3-*O*-sambubioside and 3-*O*-sophoroside. This behaviour is explained in terms of the activity of gene *Cr*.

## INTRODUCTION

STUDIES using a variety of plant species have indicated that B-ring hydroxylation and methylation, and 3- and 5-glycosylation are all terminal reactions in anthocyanin biosynthesis (Barber;[1,2] Hess; [3] Patschke and Grisebach[4]). In *Pisum*, these reactions are controlled by three Mendelian genes, *A*, basic for anthocyanin pigmentation; *B*, 5′-hydroxylation and *Cr*, 3′- and 5′-methylation. In addition, *Cr* has the pleiotropic effect of determining rhamnose as the sugar in the 3-*O*-glycoside (Statham *et al.*[5]).

Developmental studies of anthocyanin production in relation to other flavonoids, indicate that although they all share a common biosynthetic pathway, the sequence of their formation and accumulation in plant tissues may be quite independent. Generally, however, as tissues mature there is a progression from less complex to more complex molecular structures. Reznik[6] showed that white flowers of *Primula obconica* accumulated kaempferol 3-monoglucoside in the earliest bud stage, together with kaempferol 3-diglucoside in the second bud stage. In fully mature flowers, a third flavonol kaemperol 3-triglucoside, was also present. Reznik showed also that while malvidin 3-glucoside was the first anthocyanin to appear, mature flowers contained malvidin 3,5-diglucoside as well. Similarly, Hagen[7] showed a progression from pelargonidin 3-glucoside through pelargonidin 3,5-diglucoside to acylated pelargonidin 3,5-diglucoside during successive stages of bud development in a red-flowered genotype of *Impatiens balsamina*.

[1] BARBER, G. A. (1962) *Biochemistry* **1**, 463.
[2] BARBER, G. A. (1962) *Arch. Biochem. Biophys.* **7**, 204.
[3] HESS, D. (1964) *Planta* **60**, 568.
[4] PATSCHKE, L. and GRISEBACH, H. (1965) *Z. Naturforsch.* **206**, 1039.
[5] STATHAM, C., CROWDEN, R. K. and HARBORNE, J. B. (1972) *Phytochemistry* **11**, 1083.
[6] REZNIK, H. (1961) *Flora* **150**, 454.
[7] HAGEN, C. W. (1966) *Am. J. Botany* **53**, 54.

Hess[8] using three genetically homozygous lines of *Petunia hybrida*, showed that anthocyanidins appeared in the sequence cyanidin, delphinidin, peonidin, petunidin, malvidin, as floral development progressed. Thus in *Petunia*, the chemical structures of anthocyanidins present at different stages of flower development reflect the proposed biosynthetic sequence. Also, Hess found that simple glycosides of anthocyanins appeared before more complex ones.

This present paper describes developmental variation with respect to anthocyanins in flowers of four genetically pure lines of *Pisum*, and in purple flowers of *Lathyrus odoratus*. Unlike *Primula*, *Impatiens* and *Petunia*, both *Pisum* and *Lathyrus* appear to accumulate anthocyanins in the reverse order to that expected from the biosynthetic pathway.

## RESULTS

The varying complement of anthocyanins in developing flowers of *Pisum* (*B Cr*, *b Cr*, *B cr* and *b cr* genotypes), and in *Lathyrus odoratus* var Chloe, is shown in Tables 1–4 respectively.

TABLE 1. QUANTATITIVE CHANGES IN ANTHOCYANINS DURING FLORAL ONTOGENY IN *Pisum*. Genotype A Am Ar B Ce Cr (purple flowers)

| | Stage of floral development | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 |
| (a) Wing petals | | | | | | |
| Total anthocyanin ($\mu$mol) | 7 | 28 | 40 | 112 | 142 | 80 |
| Mv3R | | | | 2 | 2 | 2 |
| Mv3R, 5G | 4 | 13 | 13 | 21 | 22 | 12 |
| Pt3R, 5G | 3 | 15 | 19 | 49 | 62 | 35 |
| Dp3R, 5G | | | 8 | 32 | 47 | 24 |
| Dp3GX, 5G / Dp3GG, 5G | | | | 8 | 9 | 7 |
| Σ methylated pigments | 7 | 28 | 32 | 82 | 86 | 49 |
| Σ non-methylated pigments | | | 8 | 40 | 56 | 31 |
| Σ rhamnosylated pigments | 7 | 28 | 40 | 104 | 133 | 73 |
| Σ non-rhamnosylated pigments | | | | 8 | 9 | 7 |
| (b) Standard petals | | | | | | |
| Total anthocyanin ($\mu$mol) | | | 7 | 24 | 47 | 29 |
| Mv3R | | | | | 6 | |
| Mv3R, 5G | | | 4 | 12 | 12 | 8 |
| Pt3R, 5G | | | 3 | 12 | 21 | 14 |
| Dp3R, 5G | | | | | 8 | 7 |
| Σ methylated pigments | | | 7 | 24 | 39 | 22 |
| Σ non-methylated pigments | | | | | 8 | 7 |

### *Anthocyanidins*

In those *Pisum* genotypes which permit methylation of the anthocyanins (*Cr* types), the more complex (methylated) anthocyanidins appeared first, and unmethylated pigments were evident only at later stages of floral development. However, by stage 6 (mature flowers), in wing petals there was a predominance of part and unmethylated over fully methylated pigments. In the standard petal on the other hand, anthocyanins did not appear until about stage 4, and the sequence of accumulation thereafter reflected the progression seen

[8] HESS, D. (1963) *Planta* **59**, 567.

in earlier developmental stages (2–4) of wing petals. The total anthocyanin concentration was at all times less than in the wing petals of the same plant.

TABLE 2. QUANTITATIVE CHANGES IN ANTHOCYANINS DURING FLORAL ONTOGENY OF *Pisum*. Genotype A Am Ar *b* Ce Cr (pink flowers)

| | Stages of floral development | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 |
| Wing petals | | | | | | |
| Total anthocyanin (μmol) | 3 | 7 | 14 | 21 | 37 | 24 |
| Cy3R | | | | 4 | 2 | 2 |
| Pg3R, 5G | | | 2 | 4 | 5 | 3 |
| Pn3R, 5G | 2 | 3 | 5 | 6 | 10 | 5 |
| Cy3R, 5G | 1 | 4 | 7 | 7 | 11 | 7 |
| Cy3G, 5G | | | | | 5 | 4 |
| Cy3GX, 5G / Cy3GG, 5G | | | | | 4 | 3 |
| Σ methylated pigments | 2 | 3 | 5 | 6 | 10 | 5 |
| Σ non-methylated pigments | 1 | 4 | 9 | 15 | 27 | 19 |
| Σ rhamnosylated pigments | 3 | 7 | 14 | 21 | 28 | 17 |
| Σ non-rhamnosylated pigments | | | | | 9 | 7 |

In *Lathyrus* the flowers are much larger than those of *Pisum*, and contain more anthocyanin per flower. Also, pigment is accumulated in the standard petals of *Lathyrus* earlier than is the case for *Pisum*. However, the pattern of pigment variation and the sequence of accumulation during floral development was the same as found for *Pisum*.

TABLE 3. QUANTITATIVE CHANGES IN ANTHOCYANINS DURING FLORAL ONTOGENY OF *Pisum*

| | Stage of floral development | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 |
| (a) Genotype A Am Ar B Ce *Cr* (Crimson flowers) | | | | | | |
| Total anthocyanin (μmol) | 5 | 11 | 26 | 60 | 62 | 34 |
| Dp3GX, 5G | 5 | 8 | 19 | 45 | 51 | 31 |
| Dp3GG, 5G | | | | | | |
| Dp3DX | | 3 | 7 | 15 | 11 | 3 |
| Dp3G, 5G | | | | | | |
| (b) Genotype A Am Ar *b* Ce *cr* (Salmon flowers) | | | | | | |
| Total anthocyanin (μmol) | 4 | 10 | 17 | 26 | 37 | 23 |
| Cy3GX, 5G / Cy3GG, 5G | 4 | 10 | 13 | 16 | 24 | 15 |
| Cy3GX | | | 4 | 10 | 13 | 8 |
| Cy3G, 5G | | | | | | |

*Glycosides*

In *Lathyrus* and in *Cr* type *Pisum*, as floral ontogeny progressed complex 3-*O*-glycosides appeared, in which rhamnose is replaced by sambubiose and sophorose. These latter glycosides typify the pigments found in flowers of *cr* type *Pisum* (and in coloured pods[5]),

where neither methylation nor rhamnosylation occurs. Both wing and standard petals of *cr* type *Pisum* show the appearance of pigments with complex 3-*O*-glycosides in the earliest bud stages. Glycosylation at position 5 involves glucose only. Pigments without 5-*O*-glycoside substitution occur at late stages of floral development in all genotypes, and probably result from partial degradation of the anthocyanins.

TABLE 4. QUANTITATIVE CHANGES IN ANTHOCYANINS DURING FLORAL ONTOGENY OF *Lathyrus odoratus* var. Chloe. (Purple flowers)

| | Stage of floral development | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 |
| (a) Wing petals | | | | | | |
| Total anthocyanin ($\mu$mol) | 9 | 44 | 144 | 474 | 1140 | 778 |
| Mv3R | | | | | 23 | 23 |
| Mv3R, 5G | 9 | 37 | 80 | 161 | 365 | 234 |
| Pt3R, 5G | | 7 | 45 | 185 | 399 | 249 |
| Dp3R, 5G | | | 19 | 114 | 274 | 202 |
| Dp3GX, 5G / Dp3GG, 5G | | | | 14 | 79 | 70 |
| Σ methylated pigments | 9 | 44 | 125 | 346 | 787 | 506 |
| Σ non-methylated pigments | | | 19 | 128 | 353 | 272 |
| Σ rhamnosylated pigments | 9 | 44 | 144 | 460 | 1061 | 708 |
| Σ non-rhamnosylated pigments | | | | 14 | 79 | 70 |
| (b) Standard petals | | | | | | |
| Total anthocyanin ($\mu$mol) | 14 | 53 | 117 | 382 | 842 | 485 |
| Mv3R | | | | 15 | 59 | |
| Mv3R, 5G | 10 | 29 | 68 | 126 | 252 | 151 |
| Pt3R, 5G | 4 | 19 | 47 | 138 | 303 | 199 |
| Dp3R, 5G | | 5 | 6 | 103 | 228 | 136 |
| Σ methylated pigments | 14 | 48 | 111 | 279 | 614 | 349 |
| Σ non-methylated pigments | | 5 | 6 | 103 | 228 | 136 |

## DISCUSSION

Unlike the situation in *Primula obconica* and *Petunia hybrida*, the appearance of anthocyanins in *Pisum* and *Lathyrus* does not directly reflect the sequence of reactions in the proposed biosynthetic pathway. This situation could result from a disproportionate activity of the *Cr* gene relative to the varying rate of synthesis of the flavylium nucleus during successive stages of floral development. Thus, early in floral development, anthocyanin

TABLE 5. MEAN PERCENTAGE COMPOSITION (±S.E.) OF ANTHOCYANINS IN *Pisum*. Genotype A Am B Ce Cr (Purple flowers). *Wing petals*

| | Stage of floral development | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 |
| Mv3R | | | | 1·4 ± 0·4 | 1·6 ± 0·3 | 2 ± 0·4 |
| Mv3R, 5G | 61·2 ± 9·4 | 46·2 ± 6·3 | 33 ± 3·9 | 19 ± 0·5 | 15·5 ± 0·6 | 14·5 ± 0·6 |
| Pt3R, 5G | 38·8 ± 9·4 | 53·8 ± 6·3 | 47·2 ± 2·6 | 43·9 ± 1·0 | 44·2 ± 0·8 | 44·2 ± 0·8 |
| Dp3R, 5G | | | 19·7 ± 3·0 | 28·6 ± 0·9 | 33·2 ± 0·9 | 29·7 ± 0·9 |
| Dp3GX, 5G / Dp3GG, 5G | | | | 7·1 ± 0·7 | 6·3 ± 0·9 | 9·5 ± 0·9 |

TABLE 6. ANTHOCYANINS IN FLOWERS OF *Pisum* MUTANTS[5]

| Genotype | Phenotype | Anthocyanins |
|---|---|---|
| A Am Ar B Ce Cr | Purple flowers | Delphinidin, petunidin and malvidin, 3-rhamnoside-5-glucoside, malividin 3-rhamnoside, delphinidin 3-sophoroside-5-glucoside, delphinidin 2-sambubioside-5-glucoside |
| A Am Ar *b* Ce Cr | Pink flowers | Pelargonidin, cyanidin and peonidin, 3-rhamnoside-5-glucoside, cyanidin 3-rhamnoside, cyanidin 3-sophoroside-5-glucoside, cyanidin 3-sambubioside-5-glucoside |
| A Am Ar B Ce *cr* | Crimson flower | Delphinidin 3-sophoroside-5-glucoside, delphinidin 3-sambubioside-5-glucoside, delphinidin 3-sambubioside, delphinidin 3-glucoside-5-glucoside |
| A Am Ar *b* Ce *cr* | Salmon flowers | Cyanidin 3-sophoroside-5-glucoside, cyanidin 3-sambubioside-5-glucoside, cyanidin 3-sambubioside, cyanidin 3-glucoside-5-glucoside |

synthesis is low, and the enzymic activity controlled by the *Cr* gene is able to effect methylation of all anthocyanin nuclei. At later stages, the *Cr* gene product is apparently unable to keep pace with the rate of production of the flavylium nucleus, and unmethylated anthocyanins accumulate. This notion is supported by the temporal difference in anthocyanin production in wing and standard petals. In the standards, total anthocyanin content is less than in the wings, and whereas some unmethylated pigments are accumulated, their proportion is less than in the case of the wings. Alternatively, it might be supposed that the *Cr* gene is "switched off" at an early stage in the senescence of the flowers, so that partially and unmethylated anthocyanins are allowed to accumulate.

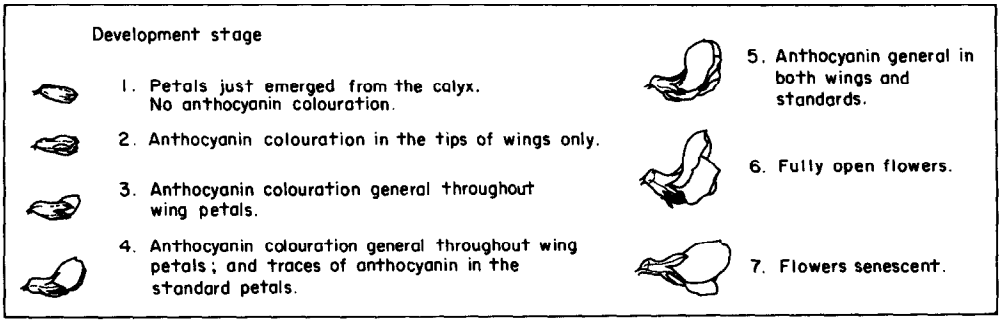


FIG. 1.

Reference has been made previously to the pleiotropic effect of *Cr*.[5] While the two biochemical functions ascribed to this gene (B-ring methylation and 3-rhamnosylation), are inseparable by genetic analysis, a temporal difference in these two functions is evident from these experiments. Thus, anthocyanidin methylation may be reduced, while 3-rhamnosylation can still proceed, so that for example, petunidin and delphinidin 3-rhamnoside-5-glucosides are accumulated. At a later stage of floral development, anthocyanins lacking rhamnose also accumulate. This observation would suggest that *Cr* is, in fact, two tightly linked loci. An alternative explanation would be that some product of *Cr* is necessary for both anthocyanidin methylation and for biosynthesis of the 3-rhamnosylglycoside and that the latter reaction has priority over methylation, when the *Cr* product is limited in amount.

## EXPERIMENTAL

*Plant materials.* Seeds from genetically pure-breeding lines were used throughout. Seeds were obtained from the Plant Breeding Institute, Weibullsholm, Sweden (S lines), and from Dr. I. C. Murfet, Botany Department, University of Tasmania (L lines). Seeds were grown in a glasshouse in containers having a 1:1 vermiculite–dolerite chips (0·5 cm mesh) solid medium. Plants were watered daily and normal Hoagland's nutrient soln applied weekly. The particular genotypes under investigation, and the anthocyanins contained in their flowers are given in Table 6. The sequence of anthocyanin development in the commercial variety of *Lathyrus odoratus* var. Chloe, (Arthur Yates and Co., Pty. Ltd., Sydney, Australia) having purple flowers was also investigated. The pigment composition was identical with that for purple-flowered *Pisum*.

*Pigment analysis.* Flowers of pure lines of both *Pisum* and *Lathyrus* were collected at various stages of development, and sorted into seven developmental stages, from the youngest colourless buds to fading, senescent flowers, as shown in Fig. 1. A pooled sample of 10 or more flowers, representing each developmental stage, was crushed and exhaustively extracted with a known volume of MeOH/1% HCl. The absorbance of these extracts was then recorded on an Hitachi 101 spectrophotometer, at 530 nm (*b* genotypes) or 540 nm (*B* genotypes and *Lathyrus*), and used to determine anthocyanin concn/flower. Wing and standard petals were treated separately. Keel petals were not investigated, since none of the *Pisum* lines used contained anthocyanin in the keel. The extracts, after concentration to a small volume, were applied as 5 cm streaks to Whatman No. 1 chromatography paper. The papers were developed overnight in BAW (*n*-BuOH–HOAc–$H_2O$, 4:1:5, upper phase). Centre strips 3-cm wide were cut from the chromatograms, thus eliminating the blurred edges of the resultant bands, and the relative density of each band in the strip determined using an EEL densitometer. In this manner, an absorbance profile showing the relative proportions of anthocyanins present in the flowers was constructed, for each of the seven developmental stages investigated. These data were then used to determine concentrations of anthocyanins in single flowers as shown in Tables 1–4.

*Sample variation.* Flower development in *Pisum* is rapid, the progression through the seven developmental stages occurring within about 48 hr in warm weather. When many plants of homozygous genotype are available for sampling, the selection and sorting of flowers into seven stages is quite accurate and there is no overlap. Within each stage, however, individual flowers show variation with respect to pigment concentration. In the method described above, the variation is largely evened out by the use of a "pooled" sample of 10 or more flowers. A statistical assessment of sample variation has been made using data obtained from six replicate determinations of pigment composition at each ontogenic stage. A typical analysis showing the average percentage pigment composition, together with estimates of the standard error (S.E.), for wing petals of *Pisum*, genotype *B.Cr*, is given in Table 5. The generally low estimates of S.E., particularly for developmental stages 4–7 indicate a low level of sample variability.

*Abbreviations.* Mv = malvidin, Pt = Petunidin, Dp = delphinidin, Pn = peonidin, Pg = Pelargonidin, 3R = 3-rhamnoside, 3R,5G = 3-rhamnoside-5-glucoside, 3GG = 3-sophoroside, 3GX = 3-sambubioside, 3G,5G = 3-glucoside-5-glucoside, 3GX,5G = 3-sambubioside-5-glucoside, 3GG,5G = 3-sophoroside-5-glucoside.

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