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# ANTHOCYANIN INHERITANCE IN PETALS OF FLAX, *LINUM USITATISSIMUM*

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**Key Word Index**—*Linum usitatissimum*; Linaceae; flax; anthocyanins; anthocyanidin 3-glucosylrutinosides; delphinidin and cyanidin 3-triglucosides; kaempferol glycosides; pigment genetics.

**Abstract**—Twelve anthocyanins have been isolated from flax: the 3-glucosylrutinosides of pelargonidin, cyanidin and delphinidin; the 3-triglucosides of delphinidin and cyanidin; a 3-diglucoside of delphinidin; the 3-rutinosides of delphinidin, cyanidin and pelargonidin; and the 3-glucosides of delphinidin, cyanidin and pelargonidin. The five triglycosides are new anthocyanins, the three with rhamnose in them being isometric with the $2^G$-glucosylrutinosides of *Rubus*. No methylated pigments were present, in spite of the earlier report of malvidin and hirsutidin glycosides in seedlings. Re-examination of the seedling anthocyanin showed only one pigment—cyanidin 3-rutinoside. Three kaempferol glycosides have been found to accumulate exclusively in petals of pelargonidin types. The 12 anthocyanins occur variously in colour mutants derived from the blue wild type; the relationship between anthocyanin structure and inheritance is briefly discussed.

## INTRODUCTION

Earlier, the leaf flavonoids of flax were examined in order to see whether there was any relationship between anthocyanin genetics in the petal and the production of related pigments in the leaf. In fact, all the colour genotypes studied contained an identical leaf pattern of eight *C*-glycosylflavonoids based on apigenin and luteolin [1]. Attention has now been turned to the anthocyanins and other flavonoids in the petals and the results of these analyses are presented in this paper.

Genetical studies of anthocyanin inheritance in flax have been carried out by Tammes [2,3] and Plonka [4,5] and the subject has been reviewed by Beard and Comstock [6]. A preliminary investigation of the anthocyanins of selected genotypes was conducted by Ponniah and Seshadri [7] but none of the pigments present was fully identified. The only previous detailed work on flax anthocyanins was that of Ibrahim and co-workers on callus tissue and seedlings. The callus pigment was identified as cyanidin 3,5-diglucoside [8] and the hypocotyl pigments of one variety of flax seedlings were found to be the 3-glucoside, 3,5-diglucoside and 3-xylosylglucoside of malvidin and the 3-diglucoside and 3,5-diglucoside of hirsutidin (7-*O*-methylmalvidin) [9].

## RESULTS

Eight colour genotypes of flax were analysed for their flavonoids and the results for the anthocyanins are presented in Table 1. In all, 12 anthocyanins based on pelargonidin, cyanidin and delphinidin were detected. At least five of these are new, namely the 3-glucosylrutinosides of delphinidin, and the cyanidin and pelargonidin 3-triglucosides of cyanidin and delphinidin. These were

Table 1. Anthocyanins identified in petals of different genotypes of flax

| Genotype | Petal colour and variety examined* | Anthocyanins present |
|---|---|---|
| $A^dN^G$ | Blue with blue veins (Hollandia) | Delphinidin 3-triglucoside (*Dp GGG*), delphinidin and cyanidin 3-glucosylrutinosides (*Dp GRG* and *Cy GRG*) and delphinidin 3-rutinoside (*Dp RG*) |
| $A^dN^Gn^G$ | Blue with white veins (Hybrid)* | *Dp GRG* and *Dp RG*, traces of *Dp GGG* and *Cy GRG* variably present |
| $a^dN^G$ | Pink (INRA 49–7:15–15) | *Cy GRG* and pelargonidin 3-glucosylrutinoside (*Pg GRG*) |
| $A^dn^f$ | Violet (Liral Duke) | Delphinidin 3-diglucoside (*Dp GG*) and delphinidin 3-glucoside (*Dp G*) |
| $a^dn^f$ | Dark pink with blue veins (INRA 47–1:8–10) | Cyanidin 3-rutinoside (*Cy RG*) and 3-glucoside (*Cy G*), pelargonidin 3-rutinoside (*Pg RG*) and 3-glucoside (*Pg G*) |
| $a^dn^fL^m$ | Lilac (INRA 49–20:21) | Cyanidin 3-triglucoside (*Cy GGG*), *Cy RG* and *Pg RG* |
| $A^dN^ff^c$ | Light blue with white veins (Stormont Motley) | *Dp GRG* |
| $A^dN^ff^{dl}$ | Light blue with light blue veins, crimped (Chubut 10) | *Dp GRG* and *Dp RG*; *Dp GGG* and *Dp GG* in traces. |

* Only one named variety is listed, but other varieties were screened and they all fell into one or other of the genotypes listed.

† A common $F_1$ hybrid between blue (e.g. Hollandia) and any white type (e.g. Wiera).

identified on the basis of spectral and $R_f$ properties and of the results of partial and complete acid hydrolysis (See Experimental).

The 3-glucosylrutinoside of pelargonidin was compared chromatographically with the 3-($2^G$-glucosylrutinoside) isolated from *Rubus* [10] but while similar in aqueous solvents, it was different in butanolic solvents. The trisaccharide obtained by $H_2O_2$ oxidation was also different from the known $2^G$-glucosylrutinose [10]. The delphinidin analogue, on partial hydrolysis, gave the 3-rutinoside, 3-glucoside and a 3-diglucoside (identical to the natural 3-diglucoside from flax, see Table 1), which from $R_f$ data appeared to be the 3-gentiobioside. Thus, the three triglycosides could be the 3-($6^G$-glucosylrutinosides); unfortunately, insufficient material was available to confirm this supposition. Lack of material also prevented full characterization of the two 3-triglucosides, but co-occurrence with the glucosylrutinosides suggests that one of the glucose–glucose linkages may be of the same nature. They are clearly different, from $R_f$ comparison, with the linear anthocyanidin 3-gentiotriosides isolated earlier from *Primula sinensis* [11].

The remarkable absence from the flowers of any methylated pigments was striking, in that glycosides of malvidin and hirsutidin had been reported in hypocotyl tissue of flax [9]. It was, therefore, decided to re-examine the pigment of flax hypocotyls, using the same flax variety Linott as employed by the earlier workers. There was only one pigment present, and this was readily identified as cyanidin 3-rutinoside by the standard procedures and confirmed by direct comparison with an authentic sample. The reason for this discrepancy with the findings of Ibrahim and his coworkers [8,9] remains to be explained.

With regard to the other flavonoids in the flowers of flax, the preliminary finding [1] that petals

of all genotypes contain the same mixture of glycosylflavones, namely iso-orientin, vitexin, isovitexin, vicenin-1 and vicenin-2 is now confirmed. These are the same compounds as occur in the leaf, except that in the leaf orientin, lucenin-1 and lucenin-2 are additionally present. Three newly discovered flavonol glycosides were found to occur in those genotypes which have pelargonidin glucosides. Two were identified as the 7-monoglucoside and 7-diglucoside of kaempferol and the third is an, as yet, uncharacterized kaempferol derivative.

More than 20 crosses were made within the genotypes listed in Table 1 and the results of analysing the progeny confirm the biochemical action of the six pigment genes are as given in Table 2. These results are in good accord with the earlier genetical work of Tammes [2,3] and Plonka [4,5] but differ slightly from those obtained by Ponniah and Seshadri [7].

## DISCUSSION

The present biochemical analysis of anthocyanins in flax petals indicates that the mode of inheritance in this plant is similar to the pattern observed in most other plants that have been investigated to date [11,12]. The occurrence of methylated anthocyanins in the leaves of a plant where the petal pigments are unmethylated would have been unusual, but our failure to confirm the presence of malvidin and hirsutidin glycosides in the hypocotyl suggests that the flax plant lacks the ability to methylate anthocyanins in all tissues.

Table 2. Biochemical effects of pigment genes in flax

| Gene | Biochemical activity |
|---|---|
| $n^G$ | Inhibits anthocyanin synthesis |
| $a^d$ | Controls hydroxylation of pelargonidin to cyanidin and of cyanidin to delphinidin |
| $f^{dl}$ | Inhibits the synthesis of 3-triglucoside and diglucoside, without affecting the production of 3-glucosylrutinoside |
| $f^e$ | Inhibits anthocyanins in the veins; in the petal, the only pigment formed is delphinidin 3-glucosylrutinoside |
| $n^f$ | Controls the synthesis of 3-rutinoside and 3-triglucoside |
| $l^m$ | Controls the synthesis of 3-glucosylrutinoside |

That the genetics of the petal anthocyanins is independent of the chemistry of the leaf and petal glycoflavones is not surprising and is a situation that has been observed in other plants [11–13]. Anthocyanins are much more closely related biosynthetically to the flavonols, than to the glycoflavones, and this is emphasized here by the fact that three kaempferol glycosides accumulate *de novo* in $a^d a^d$ genotypes which also produce cyanidin and pelargonidin derivatives. Flavonols do not accumulate in any other genotypes, although they occasionally occur in trace amounts in some; they are, however, completely absent from the delphinidin wild type from which all the other forms are presumed to be derived. A somewhat similar situation has been observed once before in *Streptocarpus hybrida*, where the pink pelargonidin types again contain kaempferol; flavonols are otherwise absent from the garden forms and also from the parental species from which they were derived [14].

With regard to the genetic control of anthocyanidin hydroxylation, the flax mutants derived from the blue wild type mutate in the usual direction to cyanidin and then to pelargonidin. However, the hydroxylation gene $a^d$ is unusual in the way it acts. Thus, when dominant, it allows the synthesis of delphinidin *and* cyanidin; when recessive, both cyanidin *and* pelargonidin are formed. One explanation of this behaviour is that it controls the enzyme catalysing the oxidation of cyanidin to delphinidin but is epistatic to a second gene concerned with pelargonidin synthesis, so that only in the recessive state is pelargonidin produced. Another gene affecting hydroxylation in $n^f$ (Table 2). When recessive, $n^f$ suppresses cyanidin synthesis and also alters the glycosylation pattern. Thus it is pleotropic in this action.

Finally, it is of interest to mention that the Mendelian nomenclature used for some of the pigment genes in flax does not fit the biochemical facts. For example, the action of the so-called "diluting" factor $f^e$ is not to dilute the pigment as such but to suppress the synthesis of particular delphinidin and cyanidin glycosides (Table 2). Another gene $f^{dl}$ present in the variety Chubut is similarly described as a "diluting" factor but

Table 3. $R_f$ values and Spectral Data for flax Anthocyanins

| Pigment* | $R_f$ (×100) BAW | BuHCl | 1%HCl | HOAc–HCl | $\lambda_{max}^{MeOH}$ | A440/$A_{max}$ | +$AlCl_3$ |
|---|---|---|---|---|---|---|---|
| *Dp GGG* | 05 | 02 | 15 | 33 | 286,538 | | +56 |
| *Dp GRG* | 13 | 16 | 44 | 51 | 279,541 | 16 | +40 |
| *Dp RG* | 18 | 08 | 17 | 38 | 273,543 | 21 | +30 |
| *Dp GG* | 06 | 01 | 23 | 39 | 277,538 | 20 | +39 |
| *Cy GGG* | 18 | 02 | 65 | 74 | 280,530 | 27 | +44 |
| *Cy GRG* | 12 | 06 | 50 | 68 | 284,528 | 23 | +43 |
| *Cy RG* | 32 | 24 | 20 | 47 | 283,526 | 23 | +37 |
| *Pg GRG*† | 21 | 07 | 69 | 76 | 278,504 | 35 | 0 |
| *Pg RG* | 52 | 38 | 30 | 53 | 270,505 | 39 | 0 |

* Data for monoglucosides omitted. For key to anthocyanins, see Table 1.

† Comparative values for pelargonidin 3-($2^G$-glucosylrutinoside) from *Rubus* were 27, 18, 67 and 76 respectively; on co-chromatography, the two pigments separated in BAW and BuHCl.

in fact it specifically inhibits the formation of the 3-triglucoside of delphinidin and must therefore be a glycosylation gene.

## EXPERIMENTAL

*Extraction and purification.* Various genotypes were obtained from the INRA collection. Fresh buds were collected in the evening in order to avoid pigment destruction in sunlight and were airdried at 40°. Material was extracted with MeOH containing 1% HCl for 4 hr and the filtered extract concentrated and then chromatographed 2-D in *n*-BuOH–HOAc–$H_2O$ (4:1:5, top layer) (BAW) and in 1% HCl. Anthocyanin spots were cut out, eluted and purified 1-D in 1% HCl and in HOAc–HCl–$H_2O$ (15:3:82) (HOAc–HCl).

*Identifications.* Anthocyanins were identified by standard procedures [15]. Flax petals are very small and even extensive collections of buds failed to give minor pigments in any quantity, after purification. Full characterisation was not always possible and in such cases reliance was placed particularly on comparative $R_f$ values. $R_f$ values are given for the purified pigments in 4 standard solvents in Table 3. The 3-rutinosides and 3-glucosides were identified by direct comparison with authentic specimens. The pigment Cy RG was further subjected to $H_2O_2$ oxidation and gave rutinose, identified by comparison with authentic material.

*Anthocyanin triglycosides.* That 5 of the flax pigments were 3-triglycosides follows from spectral and $R_f$ data (Table 3). This was confirmed by plotting $R_f$ values in 1% HCl against increasing hydroxylation. Data for *Dp GRG*, *Cy GRG* and *Pg GRG* fell on a straight line, while those for *Dp GGG* and *Cy GGG* fell on a second straight line exactly parallel to the first. Complete acid hydrolysis of *Dp GRG* gave delphinidin, glucose and rhamnose while partial acid hydrolysis gave three intermediates, *Dp GG*, *Dp RG* and *Dp G*. $H_2O_2$ oxidation of *Dp GRG* gave a trisaccharide with the following $R_G$ values: 0·77 in *n*-BuOH–$C_6H_6$–$C_6H_6N$–$H_2O$ (4:1:1:5), 0·63 in *n*-BuOH–HOAc–$H_2O$ (4:1:5), 0·58 in *n*-BuOH–EtOH–$H_2O$ (4:1:2.2) and 0·80 in PhOH–$H_2O$ (3:1). Acid hydrolysis of *Dp GGG* similarly gave delphinidin, glucose and partial hydrolysis gave 2 intermediates, *Dp GG* and *Dp G*; *Cy GGG* gave comparable results.

*Kaempferol glycosides.* These were identified as kaempferol 7-monoglucoside and 7-diglucoside on the basis of spectral measurements, with shifts, $R_f$ data and the results of acid hydrolysis. $R_f$ values (×100) were as follows for the 7-monoglucoside and 7-diglucoside respectively: 50 and 39 in BAW; 02 and 03 in $H_2O$; 14 and 32 in 15% HOAc; and 60 and 21 in PhOH–$H_2O$.

*Glycoflavones.* These were identified by the procedures described earlier [1].

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