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The genetic control of anthocyanin biosynthesis by homoeologous chromosomes in wheat

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SUMMARY

Coleoptile anthocyanin pigmentation in the hexaploid variety of wheat, Hope, is due to at least eight anthocyanins including four acylated forms. All are derivatives of either cyanidin or peonidin. Two homoeologous chromosomes, 7A and 7B, are involved in anthocyanin production. Both chromosomes carry genes that promote the synthesis of the same anthocyanins from flavonoid precursors. The roles of chromosomes 7A and 7B in anthocyanin biosynthesis and the consequences of interallelic interaction and dominance of possibly homoeologous loci are discussed.

1. INTRODUCTION

Anthocyanin pigmentation is known to occur in the allopolyploid *Triticum aestivum*, in coleoptiles, stems, glumes and grain. The anthocyanidins (aglycones) have been investigated qualitatively in coleoptiles of a number of Triticinae species, of differing ploidy levels (Van Bragt, Brouwer & Zeven, 1967) and in all except one line, cyanidin and peonidin were found. No further details on the structure of the anthocyanins in *Triticum aestivum* have been reported.

In the *Triticum aestivum* variety, Hope, intense anthocyanin pigmentation occurs in the coleoptile and in the stem of the senescing mature plant whereas in another variety, Chinese Spring, little anthocyanin pigmentation is observed in the coleoptile or stem under equivalent conditions. An analysis of substitution lines, in which pairs of Hope chromosomes have been substituted singly into the Chinese Spring genetic background (Sears, 1954) has facilitated a biochemical and genetic study of anthocyanin production in wheat.

2. MATERIALS AND METHODS

The genotypes of *Tricum aestivum* ($2n = 6x = 42$) used in these studies were the pigmented variety Hope (H), the recipient variety Chinese Spring (CS), and the substitution lines CS/H7A, CS/H7B and CS/H7D in which chromosomes 7A, 7B and 7D of Chinese Spring have been replaced by their homologues in Hope. These lines were developed by Dr E. R. Sears. To provide further material for analysis, Hope, Chinese Spring and the substitution lines CS/H7A, CS/H7B and CS/H7D were crossed in all combinations in a half-diallel.

Seeds were incubated at 25 °C for 2 days on moist filter-paper before being planted

about 0·5 cm deep in trays of vermiculite. The seedlings were grown for 5 days at 20 ± 1 °C at 2000 foot-candles light intensity. For the quantitative experiments replicate sowings of each genotype were randomized within trays.

Extraction of the pigments from the coleoptiles was in MWH,* usually 20 coleoptiles per 5 ml, for 24 h at 5 °C and was considered to be complete after re-extraction in a further 5 ml for 4 more hours.

Anthocyanins were purified by descending paper chromatography (Harborne, 1967). The crude methanolic extracts were first streaked on to HCl-washed Whatman no. 3 paper and chromatographed in BAW.* The bands were eluted in 0·1 % HCl methanol by descending chromatography, concentrated where necessary by evaporation and then rechromatographed in BAW. The resulting bands gave the pigment solution used for quantitative studies (see Fig. 1).

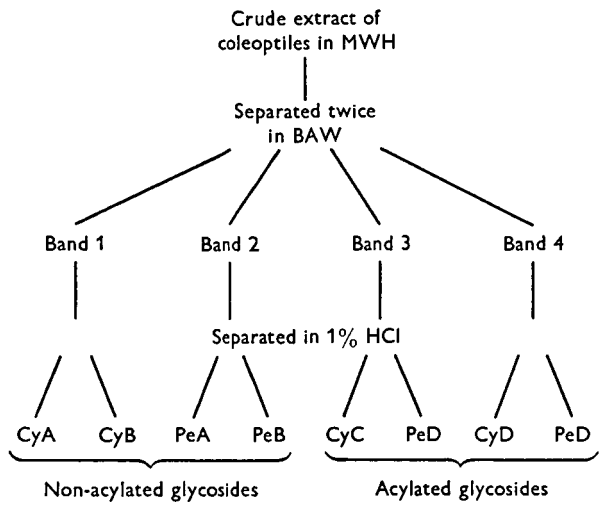

Fig. 1. Chromatographic procedures for the separation of the eight anthocyanin pigments from coleoptiles.

Identification of the purified pigments was carried out by spotting on to Whatman no. 1 paper and chromatographing in HAC: HCl,* BAW and 1 % HCl. Aglycones were isolated by hydrolysis of the anthocyanin solutions in two volumes of 4 N-HCl at 100 °C for 1 h followed by extraction in a small volume of amyl alcohol. They were identified by spotting on to Whatman no. 1 paper and co-chromatographing with known aglycones in forestal* and formic:HCl* solvents.

Acylated pigments were identified by their chromatographic R_F before and after deacylation by alkali (Harborne, 1958). The anthocyanin solutions were stored where necessary in 0·1 % HCl in methanol at -20 °C.

Where quantitative measurements were made on the extracted anthocyanins these are expressed as arbitrary units of volume × optical density at 525 mμ over

* MWH = methanol:water:HCl (60:39:1). BAW = butanol:acetic acid:water (4:1:5 top phase). 1 % HCl = water:HCl (97:3). HAC:HCl = acetic acid:HCl:water (15:3:82). Forestal = acetic acid:HCl:water (30:3:10). Formic: HCl = formic acid:HCl:water (5:2:3).

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a 1 cm light path. Quantitative comparisons of different anthocyanins are relative in the absence of extinction coefficients for each pigment.

Although some loss of pigments almost certainly occurred during our purification procedures, especially of the unstable acylated pigments (Harborne, 1958), consistent losses would not obscure genetic differences tested against replicate error.

The quantitative results were analysed using an analysis of variance and, where significant differences between lines were found, standard errors (S.E.) and least significant differences ($LSD_{5\%}$) have been calculated from the error mean square.

3. RESULTS

Observations on the substitution lines CS/H7A and CS/H7B indicated that two chromosomes, 7A and 7B, in the same homoeologous group are involved in pigment production. The remaining nineteen substitution lines showed no pigmentation under the same conditions. Measurements of optical density at 525 mμ, which

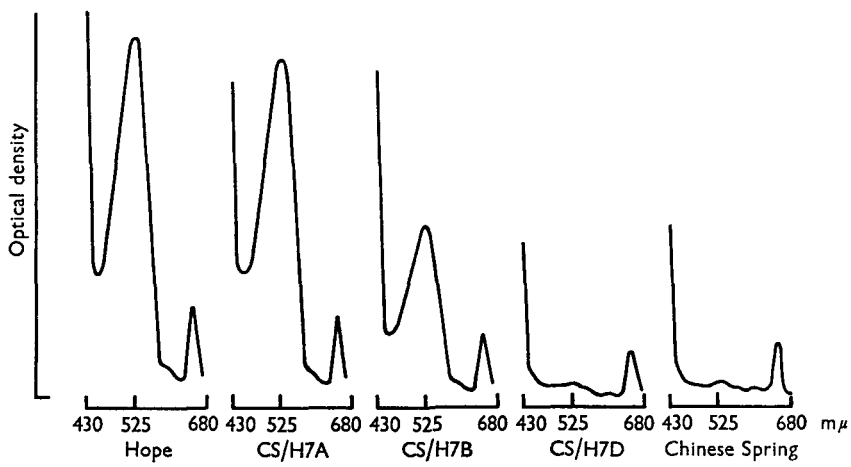

Fig. 2. Spectra of crude methanolic extracts from coleoptiles of Hope and the substitution lines of 7A, 7B and 7D Hope chromosomes in Chinese Spring. The anthocyanin peak is at 525 m.

is an absorption maximum of anthocyanins, on methanolic extracts of coleoptiles confirmed that only chromosomes H7A and H7B promote anthocyanin accumulation in Chinese Spring. The spectra illustrated in Fig. 2 show that Hope produced more anthocyanin than CS/H7A and that CS/H7A produced more than CS/H7B. Extracts from CS/H7D could not be distinguished from extracts of Chinese Spring (Fig. 2).

A qualitative analysis of the anthocyanins in Hope, CS/H7A and CS/H7B coleoptiles was undertaken.

Methanolic extracts from Hope, CS/H7A and CS/H7B coleoptiles were fractionated into eight anthocyanin pigments by the paper chromatographic procedure outlined in Fig. 1. After purification in 1 % HCl each pigment was further chromatographed using HAC–HCl and BAW as solvents. In each of these solvents

the pigments chromatographed as single bands so each pigment was considered to consist of a single anthocyanin. Extracts from coleoptiles of all three genotypes, Hope, CS/H7A and CS/H7B, produced qualitatively similar chromatograms.

Hydrolysed crude extracts from Hope, CS/H7A and CS/H7B produced only two aglycone components upon chromatography in BAW, forestal or formic:HCl solvents. These two components co-chromatographed with the anthocyanidins, cyanidin (Cy) and peonidin (Pe). Each of the eight purified anthocyanins from Hope, CS/H7A and CS/H7B were hydrolysed and the extracted aglycones were co-chromatographed with cyanidin and peonidin. The aglycone component of each anthocyanin was thus characterized.

Comparison of the R_F's in various solvents of these anthocyanins with those published by others (Harborne, 1958) for known pigments in similar solvents suggested that the glycosidic component of CyA, CyC and PeA (Fig. 1) is the glucoside. This was confirmed by co-chromatography of CyA, PeA and deacylated CyC with known anthocyanin standards.

Acylated anthocyanins are unstable in organic solvents, particularly after brief exposure to alkaline conditions, forming pigments of lower R_F (Harborne, 1958). Using these criteria the pigments, CyC, CyD, PeC and PeD were identified as acylated, although the acyl moiety(ies) was not identified.

Co-chromatography of the eight pigments from Hope, CS/H7A and CS/H7B in BAW, 1 % HCl and HAC:HCl showed that all pigments were identical in the three types of coleoptile. The lack of qualitative differences between the genotypes prompted quantitative analyses.

Using the purification scheme outlined in Fig. 1 the relative amounts of each anthocyanin and anthocyanidin in extracts from coleoptiles of Hope and the pigmented substitution lines were determined. Table 1 shows the mean of the two replicates for relative amounts of glycosides of cyanidin and peonidin in acylated and non-acylated forms. These results indicate that there is no variation in the fixing of sugars by either cyanidin or peonidin between CS/H7A and CS/H7B and, although the acylated glycosides of cyanidin and peonidin show different ratios indicating differences in acylation specificity, there is again no genotypic variation. In view of this apparent lack of genetic variation in glycosilation the results discussed below are confined to total glycosides of individual aglycones and the degree of acylation.

The mean and relative amounts of pigment of the four types in the pigmented substitution lines and the donor parent are shown in Table 2. Error was estimated from two replicates. Several differences between lines are apparent. (*a*) The total amounts of pigment in the substitution lines are not as much as in the donor parent, but CS/H7B contains a lower amount than CS/H7A. (*b*) The relative amounts of the two aglycones cyanidin and peonidin vary. CS/H7A has a Cy/Pe ratio of 1·69, compared with 1·45 for CS/H7B and the donor parent, Hope, has the greatest proportion of cyanidin with a Cy/Pe ratio of 2·86. The relative amounts of the two aglycones derived in this way agree closely with values obtained by estimating the amounts of the two pigments after hydrolysis of crude extracts. (*c*) The degree

of acylation shown by the three lines indicates a further difference between Hope (52 %) and the substitution lines CS/H7A (27 %) and CS/H7B (32 %). The acylated Cy/acylated Pe ratio in Hope was 4·75, in CS/H7A, 4·72 and in CS/H7B, 5·33, indicating that cyanidin and peonidin glycosides were acylated to the same extent in the three genotypes.

Table 1. *Relative amounts of non-acylated and acylated glycosides of cyanidin and peonidin in CS/H7A and CS/H7B*

	Non-acylated glycosides		Acylated glycosides	
Genotype	Cy A:B	Pe A:B	Cy C:D	Pe C:D
CS/H7A	43:57	35:65	62:38	81:19
CS/H7B	44:56	38:62	60:40	75:25

S.E. 3·2 $LSD_{5\%}$ 9·9

Table 2. *Mean amounts of coleoptile anthocyanin and the proportions of the glycosides of cyanidin and peonidin in the substitution lines relative to Hope*

	Non-acylated glycosides		Acylated glycosides		
Genotype	Cy A+B	Pe A+B	Cy C+D	Pe C+D	Total pigment
Hope	31 31 %	17 17 %	43 43 %	9 9 %	100
CS/H7A	34 41 %	26 31 %	15 22 %	3 5 %	78
CS/H7B	14 33 %	15 36 %	11 27 %	2 5 %	42

$S.E._{pigment}$ 2·8 $LSD_{5\%}$ 8·8
$S.E._{\%}$ 3·5 % $LSD_{5\%}$ 10·7 %

The differences between genotypes were further investigated by analysis of the coleoptile pigments from F_1 plants derived from a half-diallel cross between the donor and the recipient parents and the two pigmented substitution lines. The available seed was limited and so extractions were made of five coleoptiles in 3 ml of MWH with re-extraction in 1·5 ml. Table 3 shows the results of analysis of F_1's. Mean values of two replicates are shown for (i) total amounts of pigment and (ii) relative amounts and Cy/Pe ratios. From the total pigment extracted from each genotype it can be concluded that only partial dominance is displayed by the anthocyanin genes on chromosome 7A and 7B and a clear dosage series can be seen as the number of pigment-inducing chromosomes increases from one, in crosses of the substitution lines with Chinese Spring, to four in the parent Hope. As was observed in the parents (Table 2) the effect of 7A is greater than that of 7B. Although, in this experiment, the parent substitutions do not differ significantly, the trend is clear over arrays with the exception of the F_1, CS/H7A × Hope. The reason the latter seedlings gave such low yields of anthocyanin is not clear. The replicates agreed and the possibility of an error in labelling is remote since the Cy/Pe ratio is of the right order for a genotype that is expected to have most pigment second only to Hope itself (Table 3(ii)).

The Cy/Pe ratios vary between genotypes but are closely related to total pig-

ment and chromosome dosage, with the exception of the cross CS/H7A × H mentioned above (Fig. 3). The pattern of acylation shows significant variation over genotypes and is also associated with the dosage series of Hope 7A and 7B chromosomes (Fig. 3).

Table 3

(i) *Mean amounts of pigment in coleoptiles of F_1's, values are in arbitrary units relative to Hope*

	CS/H7A				CS/H7B				Hope			
	Cy-	Pe-	Acyl-	Total	Cy-	Pe-	Acyl-	Total	Cy-	Pe-	Acyl-	Total
CS euploid	18	23	13	54	16	20	11	47	25	25	32	82
CS/H7A	32	24	20	76	27	26	24	77	14	12	20	46
CS/H7B	—	—	—	—	23	25	23	71	19	21	30	70
Hope	—	—	—	—	—	—	—	—	27	19	54	100

S.E. 3·9 $LSD_{5\%}$ 8·3

(ii) *Percentages of anthocyanin pigments and Cy/Pe ratios in F_1's*

	CS/H7A				CS/H7B				Hope			
	Cy- %	Pe- %	Acyl- %	Cy/Pe	Cy- %	Pe- %	Acyl- %	Cy/Pe	Cy- %	Pe- %	Acyl- %	Cy/Pe
CS euploid	34	42	24	1·12	34	43	23	1·10	31	30	39	1·61
CS/H7A	42	32	26	1·69	36	32	32	1·59	31	26	43	1·92
CS/H7B	—	—	—	—	33	35	32	1·41	27	30	43	1·59
Hope	—	—	—	—	—	—	—	—	27	19	54	2·33

S.E.$_{\%\ pigment}$ 5·6 $LSD_{5\%}$ 12·0 %
S.E. Cy/Pe 0·203 $LSD_{5\%}$ 0·469

4. DISCUSSION

Sears (1954) reported that chromosome 7A of Hope carried a gene involved in coleoptile colour. We report here that Hope chromosome 7B also effects coleoptile pigmentation. A locus *Pc/pc* controlling stem colour, has also been located on the short arm of 7B (Law, 1966). The exact location of the coleoptile locus which we have called R_2/r_2 is under investigation. Genetic analyses are being carried out to determine whether *Pc* and R_2 are at the same locus and whether this locus is exactly homoeologous with that of *R/r* on 7A. The possibility of homoeology is enhanced by the report by Jha (1964) that in another hexaploid, a synthetic Spelta, coleoptile colour is determined by a gene carried on 7D. Further circumstantial evidence comes from Table 3 in which it can be seen that the interactions of *R* and R_2 with respect to both dominance and dosage are of similar nature.

The precise biosynthetic pathway of anthocyanins is unknown. It is generally accepted that an anthocyanidin precursor is formed from a colourless flavonoid (Grisebach, 1965). Anthocyanins are formed from anthocyanidins by glycosilation and acylation which are regarded as terminal steps. In both Chinese Spring and

Hope there are similar amounts of the flavone tricin and two glycoflavones, probbably isovitexin and orientin (unpublished information) so the biochemical steps controlled by R and R_2 must occur after the formation of the flavone precursor and before the formation of anthocyanidins.

The biochemical steps examined in these genotypes and their F_1 progenies include the conversion of the anthocyanidin precursor to cyanidin and peonidin and the subsequent glycosilation and acylation of these aglycones to anthocyanins.

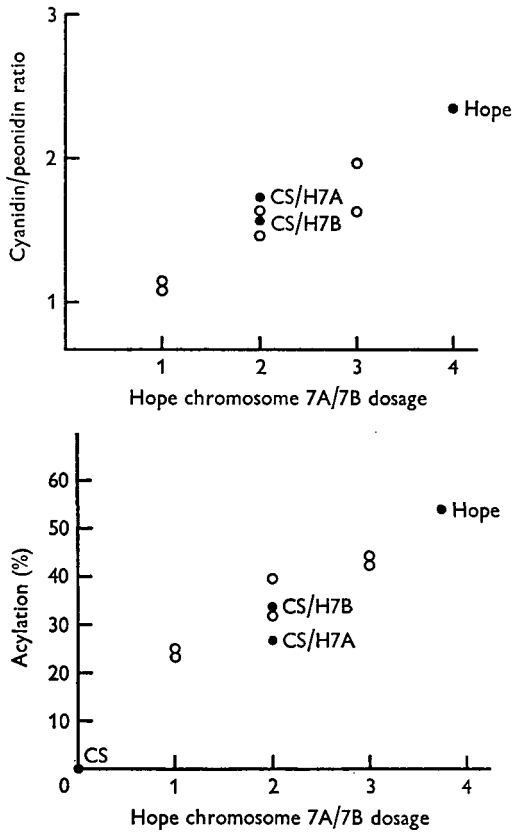

Fig. 3. The relationship of Cy/Pe ratio and the degree of acylation to Hope 7A and 7B chromosome dosage in parents (solid circles) and F_1 hybrids (open circles).

Analysis of the eight anthocyanins in the parental lines and F_1 hybrids showed differences in the total amount of pigment produced, in the relative amounts of the two aglycones formed and in the proportions of acylated glycosides. The types of anthocyanin were qualitatively similar in all pigmented lines, and in CS/H7A and CS/H7B the ratios of the non-acylated and the acylated glycosides of the two aglycones were similar. Thus we have no evidence to suggest that chromosomes 7A and 7B control anthocyanin glycosilation in wheat.

Both the Cy/Pe ratios and the degree of acylation correlate linearly with Hope 7A and 7B dosage. This is not inconsistent with a hypothesis of direct genetic

control of these parameters by Hope chromosomes 7A and 7B. However, the chromosome 7A and 7B dosage series also correlates with the total pigment produced. If cyanidin rather than peonidin is formed from the anthocyanidin precursor as more anthocyanin accumulates then the differences in Cy/Pe ratios may be under only indirect control of the R and R_2 loci on chromosome 7A and 7B. Similarly, the extent of acylation may be under only indirect genetic control of the R and R_2 loci.

Although our genetic and biochemical analyses suggest that the relevant genes on chromosome 7A and 7B control the same biochemical step they differ in apparent efficiency under the conditions described in this paper, allele R promoting more synthesis than R_2. Using other conditions more favourable to anthocyanin production, i.e. higher light intensity, lower temperature and more nutrients, we have found that Chinese Spring can produce cyanidin and peonidin glycosides. This suggests that the genes specifying the enzymes for anthocyanin biosynthesis are present in Chinese Spring and that the R/r and R_2/r_2 loci have a regulatory role possibly shifting the equilibrium between the flavonoid classes for or against anthocyanidins. Alternatively these loci may regulate the rate of breakdown of anocyanins by anthocyanases (Chichester & Nakayama, 1965) although this is less likely.

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