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INHERITANCE PATTERNS OF FLAVONOID MUTANTS OF *TRIFOLIUM SUBTERRANEUM**

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Abstract—Genetic control of flavonoid production was studied in a series of crosses between mutants of subterranean clover. Combination of gene *R*, responsible for the accumulation of cyanidin and quercetin, with a genotype homozygous for the flavonoid-inhibiting gene b^l produced a *Semi-Red* phenotype of potential value as a strain marker. Combination of two recessive genes (b^l and c^l) capable of inhibiting flavonoid production resulted in a genotype homozygous for both loci (viz. $b^lb^lc^lc^l$) which was lower in flavonoids than either parent.

INTRODUCTION

IN EARLIER studies of this series,[1-3] flavonoid patterns of several genotypes of subterranean clover (*Trifolium subterraneum*) have been compared and differences observed were interpreted in terms of genetic effects acting at various points of the pathway for flavonoid biosynthesis. In the Red Leaf mutant,[3] a single dominant gene *R* was shown to cause a gross increase in the 3′4′-dihydroxy compounds cyanidin and quercetin, whilst in mutants L858 and N4285, two simply inherited genes (b^lb^l and c^lc^l respectively) were found to greatly inhibit all flavonoid production.[2,4] A study of the interaction of gene *R* with the b^lb^l genotype seemed to be of considerable value not only to further clarify the likely sites of action of the various genes but also from the more practical viewpoint of the introduction of strain markers into the b^lb^l types whose production as oestrogen-free clovers is current in Australia.

A second biochemical genetic study in this paper describes results of crossing the mutant N4285 and L858. Because both mutants were capable of substantially reducing flavonoid production, the isolation of plants homozygous for both mutant loci (i.e. $b^lb^lc^lc^l$) was projected in the hope of isolating a derivative completely free of oestrogenic isoflavones, the major flavonoid constituents of subterranean clover.[1,4]

* Part IV in the series "Flavonoids in genotypes of *Trifolium subterraneum*". For Part III see Ref. 3.

[1] E. WONG and C. M. FRANCIS, *Phytochem.* 7, 2123 (1968).
[2] E. WONG and C. M. FRANCIS, *Phytochem.* 7, 2131 (1968).
[3] E. WONG and C. M. FRANCIS, *Phytochem.* 7, 2138 (1968).
[4] C. M. FRANCIS and A. J. MILLINGTON, *Australian J. Agric. Res.* **16**, 565 (1965).

RESULTS

Cross between Red Leaf Mutant (B^iB^iRR) *and Low Flavonoid Mutant L858* (b^ib^irr)

The F_1 (B^ib^iRr) parents contained substantial concentrations of isoflavones (more than 1% on a dry weight basis) and had red leaves. Dominance of the alleles B^i and R was thus confirmed.

In the F_2 populations two recombinants producing 'new' phenotypes were characterized visually and by isoflavone analysis. Genotypes B^iB^irr or B^ib^irr contained visually normal quantities of anthocyanin and, when analysed, normal quantities of isoflavones. These genotypes are referred to as 'Normal'. A leaf crescent was visible on the leaves. The other distinguishable recombinants of b^ib^iRR and b^ib^iRr were distinctive in being isoflavone deficient and having an anthocyanin concentration substantially less than the red leaved parent though visibly greater than b^ib^irr (L858) or B^irr (Normal) genotypes. This class was designated *Semi-Red.*

TABLE 1. INHERITANCE PATTERNS OF RED LEAF × FLAVONOID DEFICIENT MUTANT

Parents:		Red Leaf B^iB^iRR (High anthocyanin, high isoflavone)	×	L858 b^ib^irr (Anthocyanin and isoflavone deficient)	
F_1		B^ib^iRr (All Red, high isoflavones)			
		B^iR	B^ir	b^iR	b^ir
F_2	B^iR	B^iB^iRR red	B^iB^iRr red	B^ib^iRR red	B^ib^iRr red
	B^ir	B^iB^iRr red	B^iB^irr normal	B^ib^iRr red	B^ib^irr normal
	b^iR	B^ib^iRR red	B^ib^iRr red	b^ib^iRR semi-red	b^ib^iRr semi-red
	b^ir	B^ib^iRr red	B^ib^irr normal	b^ib^iRr semi-red	b^ib^irr L858
Expected phenotypic ratio:		red 9.	semi-red 3:	normal 3	L858 1
Actual figures obtained:		701:	201:	221:	74

χ^2 for deviation from the expected ratio · = 3·67 N.S.

TABLE 2. FLAVONOID CONCENTRATIONS IN F_2 PHENOTYPES

Compound	Conc. in Phenotype* Red (B^iR)	Semi-Red (b^ib^iR)	Normal (B^irr)	L858 (b^ib^irr)
Total Isoflavones	41 1	3 1	28 2	3·6
Kaempferol	1·6	1·2	1·0	1·0
Quercetin	9 4	0·2	—†	—
Anthocyanin	32·2	16·1	1·7	—

* mg/100 g fresh wt.
† — = <0·05.

Genotypes in the F_1 and F_2 populations, assuming independent assortment of the two pairs of alleles, are shown in Table 1. Quantitative measures of flavonoid content in the four distinct phenotypes are shown in Table 2.

Cross between White Seeded Low Flavonoid Mutant N4285 ($B^iB^ic^ic^i$) *and Low Flavonoid Mutant L858* ($b^ib^iC^iC^i$)

The F_1 plants ($B^ib^iC^ic^i$) from all crosses had normal isoflavone and anthocyanin content as compared to the Geraldton parent. In the F_2 population, segregation ratios were as expected for two genes independently affecting flavonoids (here indicated experimentally by isoflavone concentration) and did not differ from an expected 9:7 ratio. The isoflavone reduced plants were then further distinguished by means of their seed colour (white seed

TABLE 3. FLAVONOID PHENOTYPIC SEGREGATION IN A F_2 POPULATION OF N4285 × L858 CROSSES

Normal Isoflavone (B^iC^i)	Reduced Isoflavone (b^ib^i or c^ic^i)	
964	701	
	like N4285 (c^ic^i or $c^ic^ib^ib^i$)	like L858 (b^ib^i)
	406	295

χ^2 for deviation from an expected 9:7 ratio = 1·8455 N.S.; from an expected 9:4:3 ratio = 2·004 N.S.

TABLE 4. FLAVONOID CONTENT OF PHENOTYPES IN CROSS N4285 ($B^iB^ic^ic^i$) × L858 ($b^ib^ic^ic^i$)

	Normal (B^iC^i)	Like N4285 ($B^ic^ic^i$)	Like L858 ($b^ib^iC^i$)	Double Homozygote ($b^ib^ic^ic^i$)
	conc. mg/100 g fresh wt.			
Isoflavones				
Formononetin	94·2	24·1	9·5	6·4
Daidzein	*	*	*	*
Genistein	34·0	8·0	2·5	*
Biochanin A	104·3	7·2	*	*
Pratensein	7·2	*	*	*
Flavonols				
Kaempferol	36·0	20·1	33·2	20·0
Quercetin	8·4	4·1	7·4	1·0
Isorhamnetin	1·2	*	*	*
4′7-Dihydroxyflavonol	0·8	*	*	*
Flavones				
Luteolin	1·2	*	*	*
4′7-Dihydroxyflavone	0·6	*	*	*
Total flavonoids	288	63·5	52·6	27·4

Least difference for significance 5% level (total flavonoids) = 12·8 mg/100 g

* Present in trace amounts, <0·05 mg %.

vs. black seed). Segregation ratios found are shown in Table 3. There were no white seeded *normal* isoflavone types found.

Actual concentrations of the individual flavonoid compounds in the four phenotypes are shown in Table 4. The double homozygote ($b^1b^1c^1c^1$)* had a significantly lower flavonoid content than the other genotypes represented.

DISCUSSION

As previously shown, the presence of the dominant allele *R* can increase the anthocyanin concentration in subterranean clover leaves by a factor of 20 or more.[3] The effect of the recessive b^1b^1 gene on the other hand is to reduce all flavonoids.[2] The combination of these two genetic effects in the *Semi-Red* class of b^1b^1R genotypes produced plants low in flavonoids but increased in anthocyanin (Table 2). The previous assignment for the sites of action of these two genes (viz. b^1b^1 exerting its effect prior to the common chalcone percursor,[2] and *R* acting locally at the much later step leading to the intermediate for 3'4'-dihydroxy anthocyanin and flavonol[3]) is consistent with the present findings. It is interesting to note however, that despite the overall damping effect of the b^1b^1 locus, the effect of the *R* locus is strong enough to override this, resulting in still substantial accumulation of anthocyanin in these plants.

The *Semi-Red* genotypes are of practical importance in that a new true breeding phenotype has been produced which would be of considerable value as a strain marker in varieties bearing the b^1b^1 genes. Hitherto the incorporation of such genotypes into a programme of low oestrogen clover breeding has been restricted by their absence of leaf markings.[5]

The cross between N4285 and L858 homozygous for the recessive genes c^1c^1 and b^1b^1 respectively, is of considerable interest with the production of a new genotype, viz. $b^1b^1c^1c^1$, homozygous for both loci. Predictably perhaps, the new genotype is lower in flavonoids than either parent, and it is noteworthy that the flavonol kaempferol is again the least affected of the individual constituents (Table 4). Phenotypically this $b^1b^1c^1c^1$ genotype is white seeded like N4285. This and the fact that no white seeded *normal* type was found in the F_2 population indicate that the two phenotypic characteristics associated with mutant N4285 (viz. white seeded and low flavonoid) are not separable. It is quite possible that the mutagenic agent used to produce the mutant, viz. ethyl methanesulphonate, has affected a block of closely linked genes either chemically by ethylation or by producing minute deletions which can segregate as simple genes. In this instance it is quite possible for c^1c^1 to exert an effect both early (low flavonoid) and late (absence of anthocyanin) in the biosynthetic sequence, as postulated by us earlier.[2]

Although the double homozygote $b^1b^1c^1c^1$ was found to exhibit the additive effects of the b^1b^1 and c^1c^1 genes, the sequence in which these genes affect the flavonoid biosynthetic pathway could not be inferred from these effects. However, there is now evidence that N4285 also has effects on lignification.[6] This could be interpreted as interference at the level of cinnamic acid or earlier, this being common to both the pathways for flavonoid and lignin formation. The effect of N4285 would thus precede that of L858 which affects flavonoids only. This situation is depicted in Fig. 1.

* For procedure used in distinguishing the $b^1b^1c^1c^1$ genotype from $B^1c^1c^1$ genotypes, see Experimental.

[5] C. M. FRANCIS, *J. Dept. Agric. W.A.* **9**, 2 (1968).

[6] C. M. FRANCIS and I. D HUME (unpublished results).

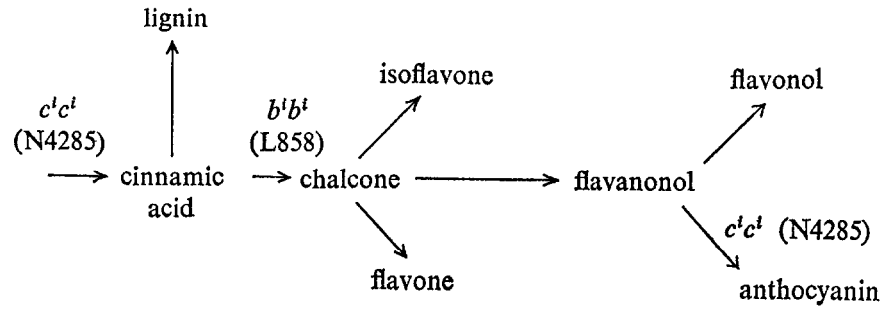

FIG. 1. POSTULATED SITES OF GENE ACTION IN GENOTYPES HOMOZYGOUS FOR LOCI b^1b^1 AND c^1c^1.

EXPERIMENTAL

Red Leaf × *Flavonoid Deficient Mutant* (*L858*) *Cross*

The parent plants were the variety Red Leaf which was homozygous for the dominant allele *R* and had normal isoflavone content. Its true breeding genotype can be signated as *RR* B^1B^1. The second parent L858 had normal anthocyanins but is isoflavone deficient and is genotypically represented as *rr* b^1b^1.

Leaf samples (12 g) of the various F_2 phenotypes were collected from at least 30 plants and extracted with ethanol. Isoflavone and flavonol contents were estimated by chromatographic and spectrophotometric means as previously described.[1]

Further leaf samples were macerated in 1% HCl in preparation for anthocyanin analysis. After 5 min in a Waring blendor, extracts were centrifuged and anthocyanin content of the supernatants were estimated spectrophotometrically (as cyanidin 3-xylosylglucoside,[3] λ_{max} 523 nm, estimated log $\epsilon = 4{\cdot}50$ (Ref. 7), equivalent to $E^{1\%}_{1cm} = 513$).

Cross between Flavonoid Deficient Mutants N4285 × L858

The mutant N4285 is distinguished by its white seeds and low isoflavones and is devoid of anthocyanin t in the leaves.[3] L858 (b^1b^1 genotype) is flavonoid deficient but has black seeds whilst its leaves have a sligh s but distinguishable anthocyanin coloration. The mutants were crossed reciprocally and the F_1 and F_2 seedlings were grouped according to their leaf appearance, seed colour and isoflavone content. Some 52 white seeded plants were backcrossed to the Geraldton parent to enable identification of plants with both 'low flavonoid' genes. From the relatively few such plants bulk samples were harvested and total flavonoids evaluated in comparison with N4285 and L858 parents using paper chromatography.[1]

Acknowledgements—The financial assistance of the Australia Wool Board is gratefully acknowledged.

[7] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 10, Academic Press, London (1967).