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Genetic Blocks in the Synthesis of Coumarin in *Melilotus alba*

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GENETIC BLOCKS IN THE SYNTHESIS OF COUMARIN

In *Melilotus alba*

H. J. Gorz and F. A. Haskins*

COUMARIN synthesis in sweetclover (*Melilotus alba*) appears to be primarily controlled by two independent pairs of genes designated as *Cu/cu* and *B/b*[1, 3]. When homozygous, the *cu* gene produces a marked reduction in the level of total coumarin while the *b* gene prevents the formation or accumulation of appreciable quantities of free coumarin in the plant. Plants of the *Cu B* phenotype contain both free and bound coumarin, plants of the *Cu b* phenotype contain bound coumarin but are very low in free coumarin, and plants of the *cu B* and *cu b* phenotypes are low in both forms of coumarin. It is reasonable to suppose that the two gene pairs affect, either directly or indirectly, certain steps in the biosynthesis of the coumarin molecule. In the terminology of the chemical geneticist, it might be said that the *cu* and *b* genes, when homozygous, block specific reactions concerned with coumarin synthesis. Such biosynthetic blocks are well known, particularly in *Neurospora crassa* and other microorganisms[5]. It is the purpose of this paper to present the results of experiments in which improved assay methods have been used to measure the influence of the two genes, *cu* and *b*, upon the level and form, respectively, of coumarin in sweetclover leaf tissue.

Materials and Methods

Twenty-five sweetclover plants of each of the four homozygous genotypes, *CuCuBB*, *CuCubb*, *cucuBB*, and *cucubb*, were used in this study. The plants of known genotype were chosen from several F_8 lines that had been derived from a single, doubly heterozygous F_6 plant. In the original cross from which this F_6 plant was derived, a *cucuBB* plant was used as the female parent and a *CuCubb* plant as the male parent. (Acknowledgment is made to Dr. W. K. Smith at the University of Wisconsin for the parental material.) Ten alfalfa plants also were assayed to provide an indication of the magnitude of fluorescence not ascribable to coumarin. Assays for free and bound coumarin were made with the use of the Beckman model DU spectrophotometer equipped with the fluorescence attachment. Young leaf tissue was used, and the procedure described by Haskins and Gorz[3] was followed.

Results and Discussion

Average levels of free, bound, and total coumarin, with standard errors, for each of the four homozygous genotypes of sweetclover and for alfalfa are shown in Table I. In Table II, levels of free and bound coumarin are expressed as percentages of the total coumarin content.

TABLE I. Average coumarin content of sweetclover leaves representing plants of four genotypes and of alfalfa leaves

	Coumarin equivalence (dry weight basis)		
	Free	Bound	Total
Genotype	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
	%	%	%
cucubb	0.02 — .002	0.20 — .022	0.22 — .022
cucuBB	0.08 — .013	0.16 — .030	0.24 — .037
CuCubb	0.02 — .001	5.13 — .268	5.15 — .268
CuCuBB	1.09 — .102	3.05 — .315	4.14 — .317
Alfalfa	0.01 — .001	0.01 — .001	0.02 — .001

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It is apparent from the data that in plants which are homozygous with respect to *cu*, the synthesis of coumarin is not completely blocked. Although the average level of total coumarin in *cucu* plants is appreciable and easily detectable, amounting to approximately 0.2 percent of the dry weight, the level is only about 1/20 of that found in *CuCu* plants. The degree of fluorescence noted in assays of *cucu* plants, however, is 11.5 times that observed for alfalfa, and it must be concluded that the *cu*-effected block in coumarin synthesis is partial rather than complete.

The *b* gene, on the other hand, appears to be highly effective in blocking the formation of free coumarin. Thus, in plants of the *CuCubb* genotype, less than one percent of the total coumarin content is in the form of free coumarin. In *cucubb* plants the proportion of free coumarin appears to be somewhat larger, amounting to about nine percent. However, for both of these genotypes the free coumarin levels listed in Table I are almost as low as the value recorded for alfalfa. It is probable, therefore, that little if any of the "free coumarin" fluorescence noted in assays involving these genotypes is actually due to coumarin. This very small amount of "free coumarin" accounts for a greater percentage of the total coumarin content in plants of the *cucubb* genotype than in *CuCubb* plants because of the low total coumarin content of the former plants. In view of these considerations, it may be assumed that the values of 0.4 percent free coumarin and 99.6 percent bound coumarin listed in Table II for the *CuCubb* genotype probably represent the upper limit for the free form and the lower limit for the bound form, respectively, in plants which are homozygous for *b*. Thus, a virtually complete blocking action is indicated for the *b* gene.

Information thus far available indicates that bound coumarin, free coumarin, and the *cu* and *b* genes are related as follows:

As indicated in this proposed pathway, bound coumarin appears to serve as a precursor of free coumarin in the plant, the *cu* gene partially blocks the conversion of unspecified precursors to bound coumarin, and the *b* gene prevents the conversion of bound coumarin to the free form. Further work on this biosynthetic pathway is planned.

The results reported in this paper have an important bearing on sweetclover breeding. One of the primary objectives in sweetclover breeding programs is the development of strains that are free of coumarin, since this chemical imparts a bitter taste to the forage and also gives rise to the toxic material, dicoumarol, which occurs in spoiled sweetclover hay and silage. Dicoumarol is the causative agent associated with the so-called "sweetclover disease" in livestock[4]. Breeding for low coumarin level requires the analysis of many thousands of plants for coumarin content. Of necessity this large-scale analysis must be done qualitatively. Qualitative fluorometric procedures[2] now available for use by the plant breeder permit the detection of very low levels of coumarin. The plant breeder should be aware that the *cu*-effected block in coumarin synthesis is partial in nature, and that some coumarin should, therefore, be expected in plants having the *cucu* genotype. An application of this knowledge may save much valuable germplasm that might otherwise be discarded as unsuitable.

It is also important to know the level of coumarin in plants of each of the various genotypes when utilizing the coumarin-conditioning alleles as genetic markers, or in studies of the physiology of coumarin synthesis in sweetclover.

Summary

The influence of the two genes, *cu* and *b*, upon the level and form of coumarin in sweetclover leaf tissue was determined by assaying 25 sweetclover plants of each of the four homozygous genotypes, *CuCuBB*, *CuCubb*, *cucuBB*, and *cucubb*. An assay of alfalfa leaves provided an indication of the magnitude of fluorescence not ascribable to coumarin. Approximately 0.2 percent total coumarin (dry weight basis) was found in plants homozygous for *cu*, which is 11.5 times the amount found in alfalfa, but only about 1/20 of the level found in *CuCu* plants. Thus, the *cu*-effected block in coumarin synthesis is partial rather than complete. However, the action of the *b* gene in blocking the formation of free coumarin is virtually complete, as shown by the extremely low levels of free coumarin in *bb* plants. The probable relationship of bound coumarin, free coumarin and the *cu* and *b* genes is shown. Important implications in sweetclover breeding, and in other studies are indicated.

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TABLE II. Levels of free and bound coumarin in four genotypes of sweetclover, expressed as percentages of total coumarin content

Genotype	Total coumarin	Percentage of total content occurring as	
		Free	Bound
	% of dry wt.		
cucubb	0.22	9.1	90.9
cucuBB	0.24	33.3	66.7
CuCubb	5.15	0.4	99.6
CuCuBB	4.14	26.3	73.7

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