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Contents of Chlorophylls *a* and *b* in Chlorophyll-Deficient Mutants of Sweetclover

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ABSTRACT

Leaf extracts from twelve chlorophyll-deficient mutants of sweetclover (*Melilotus alba* Desr.) were compared spectrophotometrically and chromatographically with extracts from normal plants to determine contents of chlorophylls *a* and *b*. Total chlorophyll contents in cotyledons and leaves of all mutants were significantly lower than those in corresponding tissues of normal plants. Chlorophyll contents were generally higher in later emerging leaves of both normal and mutant plants. Chlorophyll *a/b* ratios in first trifoliolate leaves were similar to normal in mutants ch_7 and ch_8, higher in mutants ch_6, ch_9, ch_{10}, and ch_{11}, and extremely high in mutant ch_4. A lower than normal *a/b* ratio was observed in mutant ch_{12}. The four allelic ch_5 mutants were unique in that chlorophyll *b* was not detected in any of the tissue extracts. The ch_5 mutants appear to be only the third reported instance in higher plants of a viable mutant lacking chlorophyll *b*, the previously reported cases being in barley (*Hordeum vulgare*) and *Arabidopsis thaliana*.

Additional index words: ***Melilotus alba*, Ethyl methanesulfonate.**

AMONG the most common spontaneous or induced mutations in higher plants are those that cause alterations in plant pigmentation, particularly the chlorophyll pigments (9). Although numerous chlorophyll mutants have been isolated and their mode of inheritance defined, many of these have not been characterized biochemically. The lethality of large numbers of chlorophyll mutants is a serious limitation in their biochemical analysis; it is rare that such defects can be overcome by alteration of cultural conditions. In a few instances, conditional chlorophyll mutants (i.e., those in which the expression of the mutant phenotype is controlled by environmental conditions such as light or temperature) have been isolated and used in biochemical studies (1, 8). Much information has been obtained through biochemical genetic techniques in other areas of metabolism, and biochemical studies of mutants defective in chloroplast structure, function, and pigment synthesis should be of value to plant biochemists and breeders in their efforts to improve the photosynthetic capabilities of plants.

Previous reports from this laboratory described the isolation of several chlorophyll-deficient mutants of an annual white-flowered sweetclover (*Melilotus alba* Desr.) following mutagenic treatment of seeds (7), and the subsequent genetic characterization of 12 of these mutants (3, 10). This paper reports levels of chlorophylls *a* and *b* in tissue extracts of these 12 mutants and of normal sweetclover.

MATERIALS AND METHODS

Twelve chlorophyll-deficient mutants of sweetclover were used in this study. The isolation of these mutants following treatment of germinating seeds with ethyl methanesulfonate (EMS) was described earlier (7). Eleven of the mutants behaved as monogenic recessives (3, 10). The remaining mutant phenotype was dependent upon two pairs of independent recessive genes (3); one caused a deficiency of green pigmentation, and the other caused the leaf veins to be considerably darker green than the intervening tissue (3). Complementation studies indicated that four of the 12 chlorophyll-deficient mutants involved the same functional gene (3, 10). Thus, the 12 mutant lines represented a total of nine different genes for chlorophyll deficiency and one additional gene for the prominent venation trait. The nine genes for chlorophyll deficiency were designated by the symbol, *ch*, with numerical subscripts from 4 to 12 (3, 10); the gene causing the prominent venation was designated ch_v.

Plants were grown in growth chambers at 25C and about 70% relative humidity, with continuous cool white fluorescent light at about 10,000 lux. All plants were grown from hand-scarified seeds planted in plastic trays filled with a mixture of vermiculite, soil, and sand (6:4:1 by volume) and covered with a 0.5-cm layer of silica sand. To provide uniformity in sampling, vegetative tissues were sampled when the second vegetative foliage unit above the one to be sampled had just unfolded (e.g., first trifoliolate leaves were sampled when third trifoliolate leaves had just unfolded). The tissues sampled included cotyledons, unifoliolate leaves, the first trifoliolate leaves, and, finally, the third trifoliolate leaves below the emerging inflorescence (referred to as "later trifoliolates" in the tables).

Chlorophyll was extracted by grinding the excised tissue in 80% acetone (1 ml/5 mg of tissue) with a ground-glass homogenizer. The homogenate was centrifuged at 1,500 *g* for 1 min. (to sediment chlorophyll-free particulate material), and the absorbance of the supernatant solution was determined at 663 and 645 nm. Total chlorophyll contents and chlorophyll *a/b* ratios were calculated by use of the equations of Arnon (2).

Thin-layer chromatography on powdered sugar was used to resolve chlorophyll *a* and *b* in extracts of tissues from mutant and normal plants. Chlorophyll extracts for use in chromatography were prepared in a manner similar to that used by Strain et al. (11). About 2 g of trifoliolate leaves, excised from 5-week-old seedlings, were ground in about 15 ml 80% acetone with a large ground-glass homogenizer. Homogenates were centrifuged at 1,500 *g* for 5 min. to sediment debris, and the supernatant was transferred to a separatory funnel containing an equal volume of petroleum ether. The pigments were driven into the petroleum ether upon the addition of distilled water. The petroleum ether extracts were concentrated to dryness under reduced pressure, and the dry pigments were redissolved in a minimum of petroleum ether.

Thin-layer chromatograms were prepared by spreading a 0.25-mm layer of a methanolic powdered sugar slurry (powdered confectioner's sugar containing 3% corn starch) over 20 × 20-cm glass plates. Sample extracts were applied as continuous lineal streaks along a line 3 cm from the bottom edge of the plate. The thin-layer plates, after sample loading, were developed in a solvent of petroleum ether containing 0.5% (by volume) n-propanol. Developed plates were observed under visible and ultraviolet light, and resolved chlorophyll bands were delineated with a pencil. For spectrophotometric work on the resolved pigment bands, the bands containing individual chlorophyll components were scraped off the plate, and the pigment was eluted from the sugar with anhydrous ether.

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RESULTS AND DISCUSSION

The intensity of green coloration in the foliage differed among the twelve mutants, ranging from the pale yellow-green of the ch_9 mutant to the darker green of the ch_{10} mutant. None of the mutants, however, approached the dark green of normal plants. The ch_5 ch_v mutant was distinctive because of the prominently darker veins, and the ch_8 mutant had cream-colored midribs in its trifoliolate leaves.

The chlorophyll contents (Table 1) of various mutant and normal tissues generally reflected the differences in color that were apparent to the eye. In all tissues examined, the mutants contained significantly less chlorophyll than normal plants. Among the mutants, the unifoliolate and trifoliolate leaves of ch_9 contained the least chlorophyll and those of ch_{10}, the most. Leaf tissues of ch_5 ch_v contained significantly less chlorophyll than those of ch_5. The chlorophyll contents of upper, later emerging leaves generally were higher than contents of lower leaves. This effect was not a result of senescence in lower leaves, since all leaves were sampled at about the same developmental stage.

Chlorophyll *a/b* ratios for various foliage tissues differed significantly among mutant and normal plants (Table 2). The ch_4 mutant had a nearly normal chlorophyll *a/b* ratio in the cotyledons, but the ratio was considerably higher in leaf tissues, indicating a low level of chlorophyll *b*. The ratios for ch_4 leaves also were quite variable, probably reflecting the difficulties of estimating *a/b* ratios greater than 5 by spectrophotometry (6). Chlorophyll *a/b* ratios of ch_6, ch_9, ch_{10}, and ch_{11} leaves were significantly higher than those of normal leaves, but not as high as the ratios for ch_4 leaves. Ratios for ch_7 and ch_8 leaves did not differ significantly from normal. Only ch_{12} leaves had an *a/b* ratio significantly less than normal. The ratio for ch_{12} cotyledons, however, was nearly normal. The ch_4 mutant, and to a lesser extent several other mutants, also had different *a/b* ratios for leaves than for cotyledons. The reason for this difference was not apparent. Spectrophotometric analysis of tissue extracts from the four lines carrying the ch_5 mutation indicated the absence of chlorophyll *b*; therefore, no chlorophyll *a/b* ratios were calculated for these lines.

Thin-layer chromatography of leaf extracts revealed two primary green pigments in normal plants and in all mutant lines except ch_5. These two pigments were clearly separated on the thin-layer plates, with Rf values of approximately 0.45 and 0.23. Spectral analysis of ether eluates of the two pigment bands indicated that the upper and lower bands were chlorophylls *a* and *b*, respectively. Chromatograms of ch_4 leaf extracts revealed a moderately strong chlorophyll *a* band and a *b* band that was much weaker than normal. This result agreed with the high chlorophyll *a/b* ratios shown for ch_4 in Table 2. No chlorophyll *b* band was detected in leaf extracts of any of the ch_5 lines, either by visual examination of the thin-layer plates or by spectral analysis of the appropriate zone from the plates.

Twelve chlorophyll-deficient sweetclover mutants, isolated following treatment of sweetclover seeds with EMS (7), have now been subjected to preliminary genetic and biochemical investigations. Of the 12, four (the ch_5 mutants) appear to be allelic and to lack chlorophyll *b*. The relatively frequent occurrence of this mutant type suggests that the ch_5 locus may be unusually sensitive to EMS. The ch_5 mutants can grow, flower, and produce viable seed despite their lack of chlorophyll *b*. Viable, chlorophyll *b*-lacking mutants in barley (*Hordeum vulgare* L.) (4) and *Arabidopsis thaliana* (L.) Heynh. (5) were reported some years ago; we have found no reports of such mutants in other higher plants. Recent studies on the barley mutant demonstrated that, in addition to lacking chlorohpyll *b*, the mutant also lacks a major chlorophyll-protein complex normally found in higher plant chloroplast membranes (12). Although it was first thought that the barley mutation primarily affected an enzyme involved in chlorohpyll *b* synthesis, it is now recognized that the mutation instead might affect the protein moiety of the chlorophyll-protein complex.

Available information does not permit the assignment of the primary effect of the ch_5 mutation or any of the other sweetclover *ch* mutants to a specific protein. However, we suggest that the chlorophyll-deficient mutants used in this study provide an excellent resource for the study of various aspects of chlorophyll biosynthesis, photosynthetic reactions, and chloroplast structure in sweetclover.

Table 1. Total chlorophyll contents (mg/g fresh weight) of normal (+) and chlorophyll-deficient mutant lines of sweetclover. Values shown are means of four replicates..

Line	Mutant	Cotyledons	Unifoliolates	First trifoliolates	Later trifoliolates
U389	+	1.57 a*	1.95 a	2.48 a	2.93 a
U394	ch_4	1.08 b	0.48 e	0.93 gh	0.92 g
U374	ch_5	0.47 de	0.77 d	1.22 ef	1.25 f
U395	ch_5	0.45 e	0.82 cd	1.03 fg	1.28 f
T159	ch_5	0.33 e	0.71 d	1.18 efg	1.37 f
U398	ch_5ch_v	0.35 e	0.51 e	0.76 h	0.85 g
U396	ch_6	0.62 cd	0.80 cd	1.06 fg	1.39 f
U397	ch_7	0.47 de	0.75 d	1.29 def	1.81 de
U369	ch_8	1.04 b	0.92 c	1.43 cde	1.71 e
U370	ch_9	0.76 c	0.46 e	0.31 i	0.82 g
U371	ch_{10}	0.66 c	1.13 b	1.86 b	2.31 b
U372	ch_{11}	0.70 c	0.90 c	1.56 c	2.01 cd
U373	ch_{12}	0.36 e	0.74 d	1.48 cd	2.10 bc

* Means in any one column followed by the same letter are not significantly different at the 5% level as determined by Duncan's new multiple range test.

Table 2. Chlorophyll *a/b* ratios of normal (+) and chlorophyll-deficient mutant lines of sweetclover. Values shown are the means of four replicates.

Mutant	Cotyledons	Unifoliolates	First trifoliolates	Later trifoliolates
+	3.33 c*	3.35 d	3.41 d	3.35 d
ch_4	3.43 c	17.37†	12.13†	8.37†
ch_6	3.31 c	3.80 c	3.85 c	3.90 bc
ch_7	4.38 b	3.55 cd	3.29 d	3.59 cd
ch_8	3.48 c	3.59 cd	3.35 d	3.43 d
ch_9	3.67 c	4.59 b	5.09 a	4.22 b
ch_{10}	4.24 b	4.45 b	4.40 b	4.16 b
ch_{11}	4.83 a	5.41 a	5.03 a	4.92 a
ch_{12}	3.46 c	2.53 e	2.67 e	2.83 e

* Means in any one column followed by the same letter are not significantly different at the 5% level as determined by Duncan's new multiple range test.
† Not included in the statistical test because of large nonhomogeneous error term.

REFERENCES

1. Alberte, R. S., J. D. Hesketh, G. Hofstra, J. P. Thornber, A. W. Naylor, R. L. Bernard, C. Brim, J. Endrizzi, and R. J. Kohel. 1974. Composition and activity of the photosynthetic apparatus in temperature-sensitive mutants of higher plants. Proc. Natl. Acad. Sci. USA 71:2414-2418.
2. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24:1-15.
3. Gengenbach, B. G., H. J. Gorz, and F. A. Haskins. 1970. Genetic studies of induced mutants in *Melilotus alba*. II. Inheritance and complementation of chlorophyll-deficient mutants. Crop Sci. 10:154-156.
4. Highkin, H. R., and A. W. Frenkel. 1962. Studies of growth and metabolism in a barley mutant lacking chlorophyll *b*. Plant Physiol. 37:814-820.
5. Hirono, Y., and G. P. Redei. 1963. Multiple allelic control of chlorophyll *b* level in *Arabidopsis thaliana*. Nature 197: 1324-1325.
6. Holden, M. 1965. Chlorophylls. p. 461-488. *In* T. W. Goodwin (ed.) Chemistry and biochemistry of plant pigments. Academic Press, N.Y.
7. Kleinhofs, A., H. J. Gorz, and F. A. Haskins. 1968. Mutation induction in *Melilotus alba annua* by chemical mutagens. Crop Sci. 8:631-632.
8. Millerd, A., and J. R. McWilliam. 1968. Studies on a maize mutant sensitive to low temperature. I. Influence of temperature and light on the production of chloroplast pigments. Plant Physiol. 43:1967-1972.
9. Nelson, O. E., Jr. 1967. Biochemical genetics of higher plants. Ann. Rev. Genet. 1:245-268.
10. Ronnenkamp, R. R., H. J. Gorz, and F. A. Haskins. 1975. Genetic studies of induced mutants in *Melilotus alba*. IV. Inheritance and complementation of six additional chlorophyll-deficient mutants. Crop Sci. 15:187-188.
11. Strain, H. H., J. Sherma, and M. Grandolfo. 1968. Comparative chromatography of the chloroplast pigments. Anal. Biochem. 24:54-69.
12. Thornber, J. P., and H. R. Highkin. 1974. Composition of the photosynthetic apparatus of normal barley leaves and a mutant lacking chlorophyll *b*. Eur. J. Biochem. 41:109-116.