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A Temperature-Sensitive Chlorophyll *b*-Deficient Mutant of Sweetclover (*Melilotus alba*)[1]

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ABSTRACT

The *ch4* mutant of sweetclover (*Melilotus alba*) has previously been demonstrated to be partially deficient in chlorophyll and to have a higher ratio of chlorophyll *a* to *b* than normal plants. We were able to substantiate these findings when plants were grown at 23°C and lower (permissive temperatures). However, when grown at 26°C (nonpermissive temperature) the plants produced small yellow leaves which exhibited one-twentieth the chlorophyll content of normal plants. Affected leaves did not increase their chlorophyll content when plants were incubated at permissive temperatures, but leaves which developed at the lower temperature contained increased amounts of chlorophyll. Similarly, only new leaves, not previously grown leaves, exhibited the yellow phenotype when the mutant plant was shifted from the permissive temperature to the nonpermissive temperature. Ribulose 1,5-bisphosphate carboxylase activity was decreased by half, relative to normal plants, in the mutant plants grown at the nonpermissive temperature, indicating that general protein synthesis was not greatly impaired and that the effect of the mutation was perhaps specific for chlorophyll content. HPLC analysis indicated that carotenoid content was not diminished to the same extent as chlorophyll and we have determined that the thylakoid protein kinase is not altered, as is the case for other chlorophyll *b*-deficient mutants. Experiments suggest that changes in photoperiod may be able to modulate the effect of temperature.

One type of mutation in higher plants and algae that has been widely studied results in reduced expression of Chl *b*. This pigment comprises approximately one-fourth of the Chl in most higher plants, functions in a light-harvesting capacity (*i.e.* having no known photochemical role), and is bound in pigment-protein complexes associated with both PSI and PSII. Mutants defective in the expression of Chl *b* can generally be placed in one of two phenotypes: those which have no detectable Chl *b* and which normally contain approximately half the total amount of Chl as the normal parent, or those which contain somewhat reduced amounts of Chl *b*, normally having ratios of Chl *a*/*b* >5 *versus* values of approximately 3 for the normal plants. Such mutants have been described in barley (*Hordeum vulgare*) (14), pea (*Pisum sativum*) (15, 27), maize (*Zea mays*) (16, 25), wheat (*Triticum aestivum*) (10, 12), sweetclover (*Melilotus alba*) (13, 21, 22, 26, 30), *Chlamydomonas reinhardii* (24), and other species. Such mutants are usually selected on the basis of decreased pigment content and the major physiological effect is that the fluence rate of light needed to saturate photosynthetic electron transport is increased (15, 16).

It is currently unclear how many genotypes can give rise to the Chl *b*-deficient phenotype. Chl *b*-lacking mutants of sweetclover were found by complementation analysis to be allelic whereas a Chl *b*-deficient mutant was able to genetically complement these mutants, suggesting its lesion was in a different gene (13, 26). It has also been demonstrated that thylakoid membranes of some mutants lacking Chl *b* still contain significant amounts of the Light-Harvesting Complex apoproteins which bind Chl *b* (*e.g.* barley *chlorina-f2* and *Chlamydomonas* pg 113) (4, 24) whereas other such mutants (*e.g.* sweetclover *ch5*) appear to contain very little of these polypeptides (22). Although Chl *b* mutants generally appear to have an increased thylakoid protein kinase specific activity (21, 24), some Chl *b*-deficient and -lacking mutants have a thylakoid protein kinase activity with altered affinity for ATP (21).

The effect of light, temperature, and other abiotic environmental factors on the plasticity of the photosynthetic apparatus in normal plants has been studied in a number of laboratories (for a review, see Ref. 3). Mutants lacking Chl *b* appear to be relatively insensitive to environmental conditions. However, evidence in the literature suggests that mutants only deficient in Chl *b* can have their pigment contents strongly influenced by growth conditions and that their expression of Chl *b* may be more plastic than that in the corresponding normal plants. In the Chl *b*-deficient *Oy-yg* mutant of maize the amount of Chl *b* expressed varies markedly in an inverse relationship to the light intensity during growth (16, 25). The relative proportion of pigment in the Chl *b*-containing Light-Harvesting Complex of the CD3 mutant of wheat has been reported to increase as the leaves aged (10). Such Chl *b*-deficient mutants have not been sufficiently studied to determine if conditionality or phenotypic plasticity is common to this class. While in the process of growing the *ch4* Chl *b*-deficient mutant of sweetclover for a previously reported study (22), it was observed that plants grown with artificial lighting and at ambient temperatures exhibited rather different properties over the course of the year. To determine whether this mutant might be environmentally responsive, we

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initiated a series of studies in controlled growth chambers. The results reported in this paper demonstrate that the expression of both Chl *a* and *b* in this mutant is a temperature-sensitive process.

MATERIALS AND METHODS

Seed of normal (U389, +/+ genotype), Chl *b*-deficient (U394, *ch4/ch4* genotype), and Chl *b*-lacking (U395, *ch5/ch5* genotype) sweetclover (*Melilotus alba*) were grown in a soil-vermiculite mixture for 4 to 6 weeks before use. The U395 strain is similar to the U374 Chl *b*-lacking strain previously reported on (21, 22) in that both are of the *ch5/ch5* genotype. All plants were grown in controlled environment chambers at 60% RH, 16 h photoperiod unless otherwise noted, and a photon fluence rate (PAR) of approximately 250 to 500 μE m^{-2} s^{-1}. The temperature of growth is stated in the text. Leaves were harvested, weighed, and ground in a Ten Broeck homogenizer containing 85% acetone. The final acetone concentration was adjusted to 80%, assuming 90% of the leaf weight to be water. The homogenate was centrifuged at 3000*g* for 2 min (the pellet contained no color) and the absorbance of the solution determined with a Varian DMS-90[3] spectrophotometer using a 1 nm band pass. The calibration of the monochromator was routinely checked with a holmium oxide filter. Concentrations of Chl were determined from absorbance measurements at 663 and 645 nm as described by Arnon (2). Protein concentrations were determined by the method of Dulley and Grieve (9), except that the sample absorbance was determined at 750 nm to avoid interference from Chl. Ribulose 1,5-bisphosphate carboxylase activity was measured by the method of Lorimer *et al.* (17).

Thylakoid membranes were isolated from leaves as previously described (19). Thylakoid membrane polypeptides were fractionated on urea-containing polyacrylamide gels following sample denaturation (22). Thylakoid membranes were analyzed for constituent pigment-protein complexes by solubilization with SDS and electrophoresis on polyacrylamide gels under relatively nondenaturing conditions (20). Fractionated pigment-protein complexes were excised and their absorption spectra determined in the gel slices. The affinity of the thylakoid protein kinase activity for ATP was measured as previously published (21) using a filter paper assay (19).

HPLC analysis of plant pigments was carried out with a system composed of two Waters model 510 pumps, a Waters Automated Gradient Controller, a Waters model 450 variable wavelength absorbance detector, and a Rheodyne model 7125 injector valve. The column used was a 300 × 4.1 mm Alltech Versapack C18 column with a 10 μm packing. Samples were usually applied to the column in 80% acetone, but extraction into ether, evaporation, and addition of methanol resulted in the same fractionation. The binary gradient consisted of the following: 75% methanol as initial solvent; a linear increase in methanol to 80% at 2 min; a nonlinear convex increase in methanol to 100% at 20 min (using gradient profile No. 4 on the Automated Gradient Controller); methanol maintained at 100% until 25 min; and a linear change to the initial conditions for the next fractionation. The absorbance was monitored at 450 nm. This fractionation method is modified from one devised by Eskins *et al.* (11), but results in reversed elution of lutein and Chl *b*.

RESULTS AND DISCUSSION

As described in the introductory paragraphs, our initial observation of conditionality in the Chl *b*-deficient U394 mutant sweetclover strain was that growth under ambient conditions in the laboratory produced more variability in Chl content per leaf fresh weight and in the ratio of Chl *a/b* than observed for the normal plant (U389) or for the Chl *b*-lacking U395 strain. To determine if the basis of this variability was indeed environmental, these three strains were grown in controlled-environment chambers at equivalent light intensity, photoperiod, and RH, but at different temperatures. As demonstrated in Table I, both the U394 and U395 strains have less Chl per weight of leaf than the normal U389. At all four temperatures of growth, the normal plants had approximately 2 and the U395 strain approximately 1 mg Chl per g leaf. To further substantiate that the Chl *b*-lacking strains of the *ch5/ch5* genotype did not display a decrease in pigment production when grown at 26°C, leaves from four such strains (U374, U395, U398, and T159) were analyzed after growth at 26°C and found to have an average of 1.11 (SD = 0.11) mg Chl g^{-1} of leaf. The above data are similar to previous measurements of the Chl content of these plants (29). The U394 strain, however, appeared to be temperature sensitive; that is, growth at 26°C resulted in a marked decrease in the Chl content of the plant leaves. To facilitate discussion of this phenomenon, temperatures at which the U394 strain expresses its usual phenotype (approximately 0.5 mg Chl g^{-1} of leaf and a Chl *a/b* ratio of approximately 6) will be termed permissive, whereas temperatures resulting in this newly observed decrease in pigment content will be termed nonpermissive temperatures. The ratios of Chl *a/b* in the normal U389 and the U394 mutant strain grown at permissive temperatures have been previously reported (22, 30). The ratio of Chl *a/b* in the U394 strain grown at the nonpermissive temperature was widely variable and of questionable worth. Growth at nonpermissive temperatures results in pale yellow leaves of smaller size than normal. The plants are greatly diminished in height relative to growth of the same plants at a permissive temperature or to the normal plants, and approximately one-fourth of the U394 plants die during the 6 weeks of growth.

To determine whether leaves of U394 plants grown at either the permissive or the nonpermissive temperature would be affected by exposure to the other condition, plants were grown at one temperature for approximately 5 weeks and then the chamber reprogrammed to a second temperature; the light intensity, photoperiod, and RH remained unchanged. The results of these experiments were dramatic. Leaves grown at a nonpermissive temperature remained pale yellow after the plant was switched to a permissive temperature, whereas the new leaves which developed at the permissive temperature had the usual phenotype for this mutant. Conversely, if U394 was grown at a permissive temperature and then switched to a nonpermissive temperature, only the leaves which developed and expanded at the nonpermissive temperature exhibited the pale yellow phenotype; the older leaves from the permissive growth remained healthy and as green as usual. Data for the leaves which developed during the secondary growth are shown in Table II. The U389 normal plants did not appear to be affected by the change in growth

Table I. *Pigment Content of Sweetclover Leaves as a Function of Growth Temperature*

Temperature	Pigment Content with Strain, Genotype:		
	U389 +/+	U395 *ch5/ch5*	U394 *ch4/ch4*
°C	*mg Chl a + b/g leaf*		
17	1.7	0.78	0.48
20	2.2	1.1	0.69
23	2.0	0.89	0.54
26	2.3	1.2	0.086

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture or the University of Nebraska, and does not imply its approval to the exclusion of other products that may also be suitable.

Table II. *Effect of Changes in Growth Temperature of Sweetclover on Leaf Pigment Content*

Strain	Genotype	Initial Growth Temperature	Secondary Growth[a]	
			Temperature	mg Chl g^{-1} leaf
		°C	°C	
U389	+/+	17	26	2.2
U389	+/+	26	20	2.1
U394	*ch4/ch4*	26	20	0.60
U394	*ch4/ch4*	17	26	0.16
U394	*ch4/ch4*	17	26[a]	0.93

[a] Secondary growth was with a 24 h photoperiod instead of 16 h.

Table III. *Some Characteristics of Leaves from Normal and Mutant Sweetclover Plants*

Strain	Geno-type	Growth Temperature	RuBPCase Activity	Thylakoid Protein
		°C	$\mu mol\ CO_2 \cdot g^{-1}\ leaf \cdot h$	$mg\ protein \cdot mg^{-1}\ Chl$
U389	+/+	26	1360	7.4
U395	*ch5/ch5*	26	1730	17
U394	*ch4/ch4*	26	670	42
U394	*ch4/ch4*	20	1740	21

conditions. The U394 plants from this experiment were striking, with either green leaves on the lower portion of the plant and yellowish leaves on top, or the reverse, depending on how the experiment was conducted. We also conducted one experiment shown in Table II in which U394 plants were initially grown at 17°C and then grown at 26°C in continuous light. The new leaves which developed at the higher, usually nonpermissive, temperature increased in Chl content instead of exhibiting a decrease. These data suggest that photoperiod or total fluence may be able to modulate the effect of temperature.

Surprisingly, the ribulose 1,5-bisphosphate carboxylase activities of U395 plants and U394 plants grown at a permissive temperature were markedly higher than that of the normal plant. The carboxylase activity of the U394 grown at 26°C was approximately half that of the normal plant (Table III). Since the amount of carboxylase is only reduced 2-fold in these plants while the amount of Chl is reduced approximately 20-fold, it would appear that the effect of the *ch4* mutation is somewhat specific for the pigmented components of the photosynthetic membrane. Also, since the carboxylase is composed of subunits coded in the nucleus and the chloroplast, the data imply that the mutation does not result in a general inhibition of nuclear or chloroplast-coded protein synthesis. Thylakoid membranes were isolated from these plants and the ratio of protein to Chl determined (Table III). The normal U389 and the U395 grown at 26°C had somewhat higher ratios of protein to Chl than previously reported (21), which may be due to growth at higher temperatures than in the prior study. The yield of chloroplasts isolated in the first fractionation step from U394 plants grown at 20°C, and particularly at 26°C, was less than from the U389 or U395 plants. This may be due to an increased fragility or to a decreased size of the plastids. The U394 thylakoids, with reduced Chl also had increased ratios of protein to Chl, as expected. The increased ratio of protein to pigment does not quantitatively correlate to the decrease in Chl, but the pigment exists as pigment-protein complexes and a loss of pigment is often accompanied by a loss in protein.

The protein composition of the thylakoid membranes was examined by SDS-PAGE (Fig. 1). The differences in the polypeptide pattern of U394, grown at permissive and nonpermissive

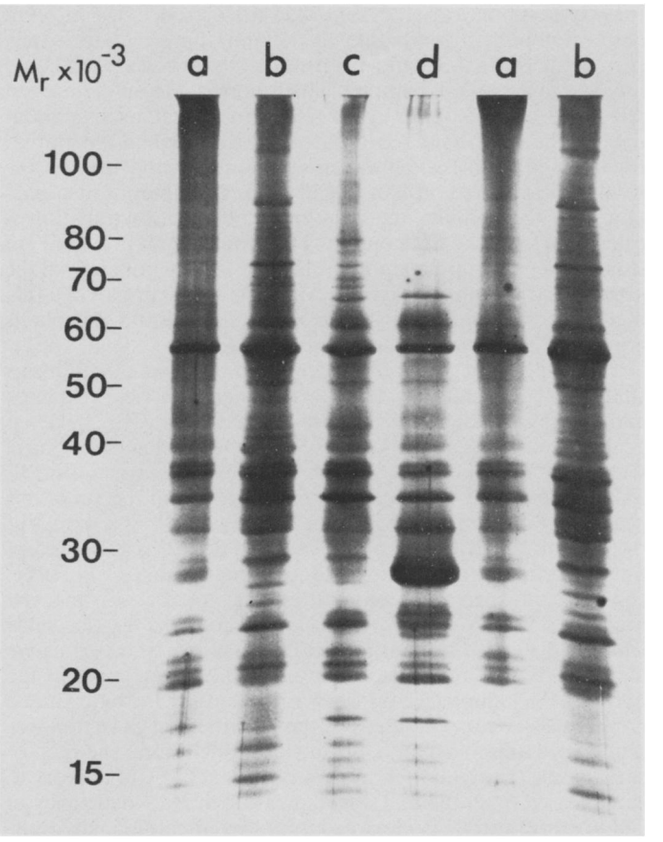

FIG. 1. Fractionation of normal and mutant thylakoid membranes by SDS-PAGE. Each lane was loaded with 12.5 μg of protein. The samples were as follows: (a), U394 grown at 20°C; (b), U394 grown at 26°C; (c), U395 grown at 26°C; and (d), U389 grown at 26°C. Following electrophoresis, the gel was silver stained.

temperatures, from that of U395 or normal U389 are not simple (*i.e.* not limited to one or two polypeptides). Both of the mutants show a marked decrease in the apoprotein of the light-harvesting complex (M_r approximately 27,000). The polypeptide pattern of the U395 *ch5/ch5* Chl *b*-lacking mutant is similar to that previously published for another *ch5/ch5* strain, U374 (22). We have previously demonstrated that at least one Chl *b*-deficient mutant has a thylakoid protein kinase with an altered affinity for ATP (21). We compared the effect of ATP on the thylakoid protein kinase activity of U394 and U389 grown at 26°C (data not shown). The response of the protein kinase activity to ATP concentration was similar in the mutant and the normal U389, which has been previously published (21). Thus, the U394 strain grown at nonpermissive temperatures does not appear to have an altered thylakoid protein kinase activity.

Thylakoid membranes of these plants were solubilized with SDS and subjected to electrophoresis under relatively nondenaturing conditions to fractionate their photosynthetic pigment-protein complexes. Analysis of the U389 plants gave the normal pattern of four pigment protein complexes (A-1, AB-1, AB-2, and AB-3) and a zone of free pigment, whereas U395 (*ch5/ch5*) produced two pigment-protein complexes (A-1 and A-2) and a free pigment band. These results were expected and similar results have been previously published (22). Fractionation of U394 grown at 20°C resolved two bands, which migrated with A-1 and AB-3, and free pigment. The spectrum of the A-1 band (not shown) was as previously published for other plants (*e.g.* 20). The spectrum of the band which migrated in the position of

the AB-3 complex (Fig. 2) suggested the presence of Chl *b* (an absorption peak at approximately 650 nm), but less than usually present in the AB-3 band from other plants (20). This could indicate that the A-2 complex which normally comigrates with AB-3 and contains only Chl *a* (25) is in greater relative abundance. The absence of AB-1 and AB-2 is similar to the pattern fractionated from the Chl *b*-deficient mutants and may be the result of the altered protein to Chl ratio. The amount of surfactant used to solubilize the membrane prior to fractionation is calculated from the Chl content. This would result in a different stoichiometry of surfactant to protein for the fractionation of the normal and mutant thylakoids. Attempts to fractionate the pigment-protein complexes from isolated thylakoids of U394 plants grown at 26°C were repeatedly unsuccessful.

The contents of Chl and carotenoid in the plants used in this study were examined by HPLC. The pigments were extracted and fractionated on a C-18 reverse-phase column (Fig. 3). Seven peaks of material absorbing at 450 nm were eluted. We have tentatively identified these peaks as follows: I, neoxanthin; II, unknown because of difficulty in obtaining useful spectra of this peak; III, violaxanthin; IV, lutein; V, Chl *b*; VI, Chl *a*; and VII, β-carotene. These identities were ascribed on the basis of absorption spectra of the fractionated material (Table IV). HPLC confirms the spectrophotometric analyses of the *ch5* and *ch4* mutants. The *ch5* mutant (strain U395) contains no detectable Chl *b* and the *ch4* mutant (strain U394) grown at a permissive temperature has an increased ratio of Chl *a*/*b*, relative to the normal U389. Similarly, no Chl *b* is evident in U394 grown at a nonpermissive temperature, but the sensitivity of detection was diminished and this may not be a valid conclusion. The ratio of carotenoids (particularly lutein and carotene) to Chl appears to be increased in all the mutants, indicating that the deficiency in Chl is not directly accompanied by a loss of carotenoids. Deficiency in carotenoids can result in a decreased Chl content (23), but that does not appear to be the case in these sweetclover mutants.

To corroborate that the pigment content of this mutant is sensitive to photoperiod or total fluence as well as temperature, plants were grown at 26°C with increased photoperiod (Table V). Growth under these conditions had little effect on the U389 or U395 plants. However, the effect on these growth conditions on the U394 strains was marked. Growth of U394 plants at 26°C with the usual 16 h photoperiod resulted in the pale phenotype normally observed at this temperature, whereas growth with a 24 h photoperiod resulted in a 10-fold increase in pigment content. Thus, an increased photoperiod is able to forestall dramatically decreased pigment content at a normally nonpermissive temperature. To test whether reduced photoperiod would affect plants grown at a normally permissive temperature, plants were also grown at 23°C with a photoperiod of either 8 or 16 h. Again, there was little effect on the U389 or U395 plants. Growth of U394 at 23°C with a 16 h photoperiod, as expected, produced plants with pigment levels comparable to growth at permissive temperatures, whereas growth at the same temperature with an 8 h photoperiod resulted in a dramatic decrease in pigment content. These results suggest an interplay between temperature and either photoperiod or total fluence in the dual phenotypes expressed in the *ch4* mutant. Extremely pigment-deficient leaves can be produced by either increasing the growth temperature or decreasing the photoperiod. The usual phenotype for the *ch4* mutant, moderate pigment content and a partial deficiency in Chl *b*, can be produced at normally nonpermissive temperatures by the use of continuous light. This phenomenon may explain why earlier work on this mutant (30) did not report a temperature sensitivity even though the plants were grown at 25°C; these plants were grown under continuous light.

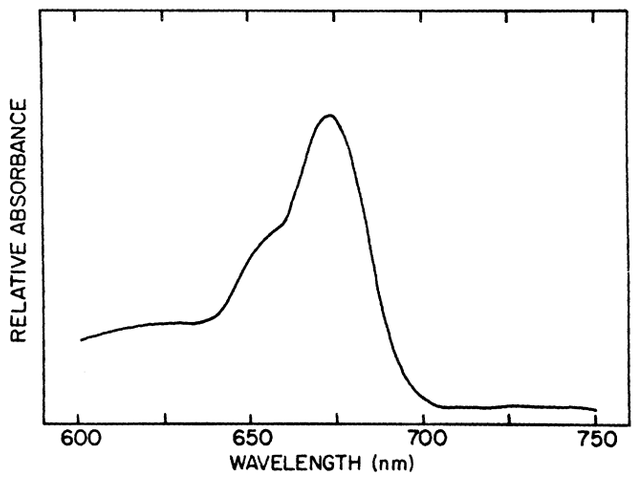

FIG. 2. Room temperature absorption of pigment-protein complex fractionated from thylakoid membranes of U394 grown at 20°C which co-migrated with the AB-3 band from normal thylakoid membranes.

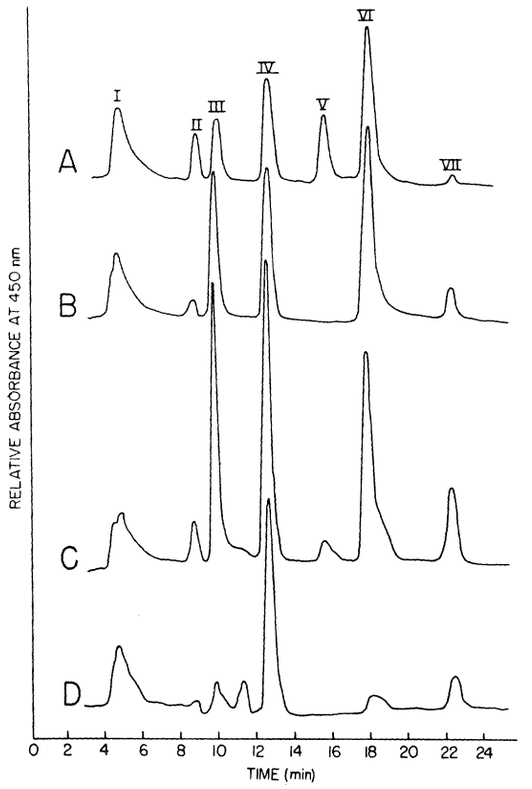

FIG. 3. Elution profiles of pigments from normal and mutant sweetclover plants. Pigments to be fractionated were extracted from the following samples: A, U389 grown at 26°C; B, U395 grown at 26°C; C, U394 grown at 20°C; and D, U394 grown at 26°C. Peaks are designated by roman numerals and identified in Table IV.

It is well documented that most normal plants have a range of temperatures in which they can grow and produce normally pigmented leaves; growth outside of this temperature range can result in decreased pigmentation (6, 28). There have been reports of mutants with greatly reduced pigment content when grown at temperatures which do not have an effect on normal plants. One such mutant is the *tigrina*-o[34] of barley described by Casadoro *et al.* (7). Mutants of the *tigrina* phenotype exhibit alternate transverse green and necrotic bands when grown under a light-dark cycle. This mutant has a lesion in the control mechanism usually

Table IV. *Spectral Identification of Pigments Fractionated by HPLC*

The observed absorption maxima of the isolated peaks were compared to reported values.

Peak	Approximate Retention Time	Identity	Observed Maxima	Solvent	Reported Maxima	Ref.
	min		*nm*		*nm*	
I	4.75	Neoxanthin	410, 436, 465	Ethanol	415, 438, 467	8
III	9.75	Violaxanthin	416, 438, 468	Ethanol	417, 440, 469	8
IV	12.5	Lutein	421, 444, 473	Ethanol	422, 445, 474	8
V	15.75	Chl *b*	457, 645	Ether	453, 642	18
VI	18	Chl *a*	431, 662	Ether	430, 660	18
VII	22.25	Carotene	425, 448, 472	Ethanol	427, 449, 475	8

Table V. *Pigment Content of Sweetclover Leaves as a Function of Growth Temperature and Photoperiod*

Temperature	Photoperiod	Pigment Content with Strain, Genotype:		
		U389 +/+	U395 *ch5/ch5*	U394 *ch4/ch4*
°C	*h*	*mg Chl g^{-1} of leaf*		
26	24	2.3	1.6	0.94
26	16	2.8	1.4	0.07
23	16	2.4	0.95	0.58
23	8	2.2	0.81	0.12

operating in the dark to restrict aminolevulinate formation. The result is excessive accumulation of Pchlide in the dark and is also accompanied by partial blocks in cyclic carotenoid biosynthesis. The mutants have decreased amounts of β-carotene and greatly increased amounts of lycopene, the immediate acyclic precursor of the cyclic carotenoids. Pchlide in high concentrations in this mutant acts as a photosensitizer, causing photodynamic destruction which is exacerbated by the decrease in β-carotene. Other characteristics of this mutant include the appearance of a new spectral form of Chl at 743 nm, photodestruction of chloroplast ribosomes, and normal ratios of Chl *a*/*b* when grown under white light. The mutant phenotype was expressed in plants grown under 23°C, but was partially relieved by growth at 30°C. The sweetclover *ch4* mutant has properties very different from those of the *tigrina*-o[34] mutant. The sweetclover mutant is sensitive to increased temperatures, not lower temperatures, and while it exhibits chlorosis, no necrosis is evident at the nonpermissive temperature. Furthermore, the sweetclover *ch4* mutant contained no detectable lycopene, no detectable Chl absorption at 743 nm (data not shown), had elevated ratios of Chl *a*/*b* when grown at the permissive temperatures, and had functional chloroplast ribosomes as judged by the amount of ribulose 1,5-bisphosphate carboxylase activity. While more work will be required to rule out a photodynamic mechanism for the mutant phenotype, it appears that the *ch4* sweetclover mutant is quite different from the *tigrina*-o[34] mutant.

Other temperature-sensitive barley mutants have also been reported. Smillie *et al.* (29) described a number of temperature-sensitive, recessive nuclear gene *viridis* mutants. The mutant phenotype was expressed at high as well as low temperatures and resulted in pale yellow and pale green plants. The molecular defect was found to be at some early step in chloroplast biogenesis. Since these barley mutants were grown under continuous illumination, it is not known if they respond to photoperiod as well as to temperature. The phenotype of these barley mutants resemble the *ch4* sweetclover mutant, except that the barley mutants all had normal ratios of Chl *a*/*b* when grown at permissive temperatures, whereas the *ch4* sweetclover mutant has an elevated Chl *a*/*b* ratio.

The *ch4* sweetclover mutant is the third mutant we are aware of which is partially deficient in Chl *b* and which exhibits phenotypic plasticity with respect to Chl content. It is unclear at present why these two traits should be thus linked. However, the Chl *b*-deficient phenotype expressed in U394 (*ch4/ch4* genotype) is clearly different from that in the Chl *b*-lacking U395 strain (*ch5/ch5* genotype). Clearly, only the former mutant is strongly affected by the growth temperature and the light regime. Since leaves which have developed and expanded at either a nonpermissive or permissive temperature are not greatly altered by transition to the other condition, it appears that some developmentally regulated process is involved in expression of the phenotype, perhaps the thermal equilibrium of some light-sensitive component or the ability to stabilize newly synthesized photosynthetic pigment-protein complexes (1, 5). Further work will be required to characterize the molecular basis of this mutation, but it is possible that study of the *ch4* sweetclover mutant will prove useful in the elucidation of the sequential processes involved in, and controls governing, the development of the photosynthetic apparatus.

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