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Studies on a Maize Mutant Sensitive to Low Temperature I. Influence of Temperature and Light on the Production of Chloroplast Pigments

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Abstract. **A mutant inbred line of *Zea mays* L. (M11) in which chlorophyll accumulation is particularly sensitive to low temperature is described. Under natural light conditions the chlorophyll content of seedlings is negligible below 17° but is normal at high temperature. Seedlings of M11 can synthesize chloroplast pigments at 16° but at a rate slower than normal. When photo-oxidation is minimized, chlorophyll accumulates, and seedlings can photosynthesize efficiently at low temperature. The primary site of low temperature sensitivity in M11 is the shoot apex where new leaves are developing and undergoing rapid cell expansion. It seems that there is impaired development and associated impaired function of chloroplasts in M11 grown at low temperatures which sensitizes them to rapid photo-oxidation in the light.**

Most tropical plants, including the cultivated forms of maize (*Zea mays* L.), are not well adapted to growth at temperatures much below 15°. Non-freezing temperatures in this range have also been found to increase the mutant expression in various chlorophyll-deficient maize and barley mutants (7, 12, 14).

A mutant inbred line of maize (M11) which is unusually sensitive to low temperature has been described by McWilliam and Griffing (10). This mutant has a high temperature threshold for chlorophyll accumulation (approx. 17°) and below this temperature, although the chlorophyll content is essentially zero, development and morphology of the plants are normal and seedlings die only when seed reserves are exhausted.

Temperature-sensitive chlorophyll mutants of this type have been observed in maize inbreds under field conditions during the early spring (Brink, personal communication) and in appearance they resemble tropical grass seedlings which are slow growing and often chlorotic at low temperature (8).

The maize mutant M11 has been examined to determine the influence of temperature on chlorophyll accumulation and chloroplast development, and in this paper we describe the role of temperature and light in the production of chloroplast pigments.

Materials and Methods

The temperature-sensitive mutant line (M11) of maize (*Zea mays* L.) used in these experiments was derived from a commercial inbred line (38-11) introduced from U.S.A. Comparisons have been made between M11 and an unrelated inbred (K4) from Missouri, U.S.A., which has a normal response to temperature. M11 has also been compared with the F_1 hybrid (M11 x K4) and its reciprocal (K4 x M11), which are normal and indistinguishable in their performance.

Growth Conditions. Plants were grown in the Controlled Environment Research Laboratory at Canberra (13). Experiments were conducted using small seedlings grown in an equal mixture of perlite and vermiculite and irrigated with half strength Hoagland's nutrient solution. When grown in the light, seedlings were harvested at the time of appearance of the third leaf. In the dark-grown material, plants were harvested when the first leaf blade was approximately 5 cm and had extended 3 cm beyond the ruptured tip of the coleoptile.

To measure the effect of temperature on chlorophyll accumulation, seedlings were germinated and grown over a range of constant temperatures from 12° to 27° in naturally lit growth chambers. The photoperiod was 16 hr consisting of 8 hr natural light, and 8 hr of low intensity incandescent light (40 ft-c).

All other experiments were conducted in artificially lit growth cabinets at either low (16°) or high (27°) temperature in continuous light at low intensity (300 ft-c), supplied by Phillips TL MF 125 W fluorescent tubes and incandescent lamps. Low light intensity was used to reduce the photo-oxidation of chlorophyll which occurs in maize grown at low temperature (11).

To separate the effect of low temperature on the expanding leaves (cell expansion phase) from its effect on the developing apex (cell division phase), seedlings were grown in an aerated, temperature-controlled, nutrient solution by supporting them in a thick polyurethane sheet which formed an insulated shield over the top of the nutrient tank. Seedlings were initially grown in the dark, then transferred to a growth cabinet, and positioned so that the apex and leaves were above the seal, in the light and at

the temperature of the cabinet; or the apex was below the seal at the temperature of the roots, with the leaves remaining above. By varying the temperature of the tank and the cabinet independently between 16° and 27° a total of 6 temperature and plant combinations were achieved.

Comparisons of the rates of synthesis of the various chloroplast pigments were conducted with young seedlings grown in the dark at either 16° or 27°. Manipulations of the plants were carried out in dim green light ineffective in transforming protochlorophyllide.

Estimation of Chloroplast Pigments. Pigments were extracted into 80 % acetone. In tissue exposed to light, the concentrations of chlorophylls *a* and *b* were determined according to Arnon (1).

For the assay of pigments in material grown in darkness or with a very low concentration of pigments, tissue above the apex was harvested and the tips of leaves, representing cells laid down in the embryo, were discarded. The remaining leaf tissue was weighed and ground in 85 % acetone to give 100 to 200 mg fr wt of tissue per 10 ml of acetone. Pigment concentrations were obtained with a spectrofluorimeter capable of recording absolute emission spectra (3). The method did not distinguish between chlorophyll *a* and chlorophyllide *a*, or between protochlorophyll and protochlorophyllide. Results have therefore been expressed as chlorophyll *a* and protochlorophyll. Chlorophyll *a* and protochlorophyll were excited at 430 mμ, chlorophyll *b* at 458 mμ. Chlorophyll *a* was measured at its emission peak at 670 mμ. Protochlorophyll and chlorophyll *b* were determined at 627 mμ and 648 mμ respectively. The fluorescence method was calibrated with purified pigments. Chlorophylls *a* and *b* were purified according to Strain (16), protochlorophyll according to Smith and Benitez (15). If necessary, the leaf extracts were diluted to an absorbancy about 0.1 at 430 mμ or 458 mμ. Under these conditions, the fractions of exciting light absorbed by chlorophylls *a* and *b* and protochlorophyll were proportional to their respective concentrations.

To estimate total carotenoids, the acetone extract, after addition of about 10 μl concentrated ammonium hydroxide, was shaken with 1 volume of ether, and the mixture then shaken with 1 volume 4 % NaCl. The ether layer was dried over anhydrous Na_2SO_4. Carotenoid concentrations (μg/ml) were obtained by dividing the absorbance values at 442 mμ by the factor 0.24 (2).

Photosynthesis and Translocation. Rates of photosynthesis in single leaves of M11 and K4 which had developed at 16° were measured by infra-red gas analysis. Chlorophyll accumulation was achieved by exposure to 10 days of intermittent light (flashing on a 30 min cycle) followed by 20 days of continuous low light (300 ft-c). Photosynthesis was measured in an assimilation chamber at 16° with a light intensity of 2350 ft-c and an air flow of 1 liter/min.

To compare rates of translocation in the same material, $^{14}CO_2$ was fed to the leaves over a period of 3 hr and the pattern of distribution of the ^{14}C label in the shoot and roots determined (17) after 72 hr under continuous low light at 16°.

Results

Temperature and Chlorophyll Formation. The influence of temperature on the chlorophyll content of seedlings M11, the unrelated inbred K4 and F_1 (M11 x K4) is illustrated in figure 1. There was little difference in the chlorophyll content of K4 and the F_1 over the temperature range 12°–27° (fig 1) although in these young seedlings the rate of chlorophyll accumulation was clearly temperature dependent. Below 20° the chlorophyll content declined steadily with decreasing temperature, and at 12° seedlings were small and chlorotic.

Chlorophyll accumulation in M11 was also temperature dependent, but the threshold was about 4° to 5° higher. Below 17° the chlorophyll content of M11 was very low, and was confined to the tips of the first 3 leaves. Above 17° the level was consistently lower than in the normal lines. Delayed greening at higher temperatures in M11 was more pronounced in the first formed leaves, but in all cases the differences were reduced with time. For example, at 20°, when harvested at the third leaf stage, the chlorophyll content of M11 was 56 % that of the F_1, but 10 days later it was 80 %.

Genetics of Low Temperature Sensitivity. The genetic control of temperature sensitivity in M11, based on an analysis of the chlorophyll content of seedling grown at 16°, was studied in F_1, F_2, and backcross populations involving the inbred K4. An estimate of the frequency of similar mutations in maize was obtained by screening a large number of maize inbreds (350) representing both American and

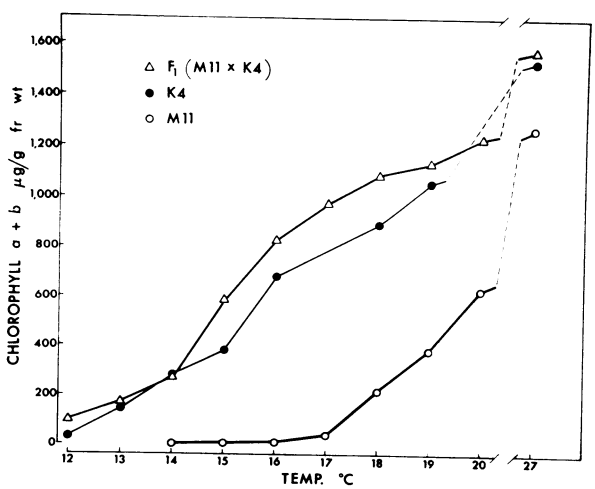

FIG. 1. Influence of temperature on the chlorophyll content of the maize inbreds (M11 and K4) and their F_1 hybrid. Seedlings were grown in naturally lit growth cabinets and harvested on appearance of the third leaf.

Australian material. Only 3 mutants similar in phenotype to M11 were found. In addition to these "white" mutant lines, there were also a large number of lines in which the chlorophyll concentration was markedly lower than the green inbreds of the K4 type.

Table I. *Segregation for Temperature Sensitivity in Crosses Involving Mutant M11 and Normal Inbred K4*

Seedlings were grown at 16° under low light intensity (300 ft-c) and rated at the third leaf stage.

Genotype	Segregation		
	Green	Pale green[2]	White
M11			360
K4	330		
F_1 (M11xK4)	385		
F_1 (K4xM11)	380		
F_2 (M11xK4)[1]	295	102	16
B.C. (M11) x (M11xK4)[1]	141	150	89

[1] Data for various reciprocals combined.
[2] Classification not homogenous includes range from very pale to near normal green.

Segregation in the progeny of crosses between M11 and K4 are given in table I. Both reciprocal hybrids were green, and there was no evidence of extra-nuclear inheritance. The F_2 and backcross progeny contained both fully green and white seedlings plus an intermediate class which was scored collectively as pale green. Comparison of the ratios obtained with those expected for the common types of multihybrid 3-class segregations (4) favor a model involving the segregation of 3 or more genes which contribute to the restoration of chlorophyll formation at low temperature. Variation in gene expression and interaction between loci is suggested to account for the reduced recovery of parental ecotypes. In at least 2 mutant inbreds the same loci were involved since the progeny from the cross M11 x 61A-4 were all mutant at low temperature.

Synthesis of Chloroplast Pigments. Chlorophyll accumulated in M11 at 16° in low light, if seedlings were first grown in the dark at 27° (table II). Greening under these conditions was confined to the leaves which had developed at high temperature.

Table II. *Chlorophyll Production in Low Light at 16° Following Etiolation at 27°*

Seedlings of M11 were grown in the dark at 27° until the first leaf was approx. 5 cm long and then transferred to low intensity light (300 ft-c) at 16°.

Line	Chlorophyll $a + b$	
	3 days	6 days
	μg/g fr wt	
K4	612	2016
M11	485	1617
M11/K4 %	79	80

These results also suggest that the small amount of chlorophyll present in the leaf tips of M11 at low temperature is a consequence of their development at high temperature in the embryo during grain maturation.

During greening at low temperature, chlorophyll is susceptible to photo-oxidation in light of high intensity (11). Once the chlorophyll is formed and complexed in the chloroplast, however, it appears to be stable at 16° as shown by the results in table III. Seedlings of M11 greened in the light at 27° remained green when transferred to 16°, and over a period of 14 days showed an increase in total chlorophyll comparable with the F_1. This suggests that once chloroplasts are formed, further development can proceed at 16°.

Table III. *Stability of Chlorophyll (Formed at 27°) When Transferred to 16°*

Seedlings were grown at 27° until the appearance of the third leaf and the chlorophyll content determined. Plants remained at 27° or were transferred to 16°; 14 days later chlorophyll content of the first 3 leaves was determined.

Line	Chlorophyll $a + b$ at time of transfer	Chlorophyll $a + b$ 14 days later	
	27°	27°	16°
	μg/g fr wt	*μg/g fr wt*	
F_1 (K4 x M11)	1270	1614	1439
M11	913	1484	1112
M11/F_1 %	72	92	77

The effect of low temperature on the shoot apex as distinct from its effect on the expanding leaf, or on the leaf and apex together, is illustrated in table IV. The chlorophyll content of M11 has been expressed as a percent of the F_1 (K4 x M11) which was grown over the same series of temperature combinations. The second leaf was used for the chlorophyll assay, as much of its development, and certainly that of the lower half of the blade occurred during the temperature treatments.

In M11 the temperature experienced at the shoot apex, a region of dividing cells, largely determined the amount of chlorophyll accumulated in the second leaf. When the apex was maintained at 27°, the base of the second leaf was green, irrespective of the temperature at which it developed. On the contrary, when the apex was at 16°, the base of the second leaf was essentially devoid of chlorophyll. The upper half of the second leaf which had partly differentiated before exposure to the various temperature treatments behaved in a different fashion. It accumulated chlorophyll slowly when exposed to 16°, because of its partial development at 27°, whereas at 27° substantial greening occurred. In the mutants this recovery at high temperature was restricted to immature leaves, and was considerably slower than in normal seedlings. Varying the root temperatures in these experiments affected growth rate, but had no influence on chlorophyll accumula-

Table IV. *Effect of Temperature During Early Development on Chlorophyll Content of the Second Leaf*

Seedlings of M11 and the F_1 (K4xM11) were grown in the dark at 16° and transferred to light (300 ft-c) when the first leaf was 5 cm long. Seedlings were supported in the polyurethane seal of a temperature-controlled tank. Seedlings were placed so that the leaves and apex were above the seal, and at the temperature of the cabinet, or the apex was below and the leaves above. The temperatures of the tank and cabinet were varied independently.

		Position of Plant					
	27°	Shoot Apex Root	Shoot Apex	Root Apex	Shoot	Root	
Temp	16°		Root	Shoot	Apex Root	Apex Shoot	Shoot Apex Root
Chlorophyll content of 2nd leaf of M11 as % of F_1	Upper half	97	86	21	65	3	2
	Basal half	99	93	91	9	6	1

tion. Also, maintaining the seed (endosperm) at either high or low temperature was without effect.

The rate of synthesis of protochlorophyll was compared in M11, and in the F_1 (K4 x M11) at both 16° and 27°. Dark grown seedlings were exposed to 10 min of red light (total energy 1000 μw/cm^2, maximum emission at 650 mμ) to convert protochlorophyll to chlorophyll *a*, and by measuring the amount of protochlorophyll remaining immediately after the light treatment, and again after 1 hr in the dark, an estimate of the rate of resynthesis of protochlorophyll was obtained. At 27° the rate in M11 was only slightly less than in the F_1, and the total protochlorophyll present after 1 hr was also comparable. At 16° the amount of protochlorophyll was considerably reduced, particularly in M11, in which the amount formed was half that found in the F_1 (table V).

Similar estimates over a longer time period were obtained indirectly by measuring the amount of chlorophyll accumulated following brief exposures to high light intensity at 16°. To avoid photo-oxidation of pigments during the light treatments, intermittent light from an electronic unit activated every 30 or 60 min was used. The flash duration was 1 msec and the color temperature 5600°K.

The results of 3 such experiments are reported in table VI. Photo-reduction was achieved with an electronic flash triggered once per hr for the 1 and 5 hr treatments, and once per 30 min for the 2 and 4 day treatments. Under these conditions chlorophyll *a* accumulated but no chlorophyll *b* could be detected. Although K4 and the F_1 are not equivalent controls, and seedling size varied slightly between experiments, the reduced rate of chlorophyll production in M11 is quite apparent, and reflects the rate of protochlorophyll synthesis during this early stage of development. Application of delta-aminolevulinic acid to the leaves of M11 before exposure to intermittent light at 16° caused the accumulation of protochlorophyllide as has been observed with other plants (6).

Despite the reduced rate of chlorophyll synthesis in M11, the amount accumulated after 96 hr of intermittent light was quite appreciable, and sufficient to cause visible greening of leaves, which continued when the plants were transferred to low light intensity at 16°.

Table V. *Rate of Protochlorophyll Synthesis at High and Low Temperatures*

Seedlings were grown in the dark at 16° or 27° until the first leaf was approximately 5 cm long. The rate of protochlorophyll synthesis was determined from the difference between protochlorophyll content following exposure to red light (10 min, total energy 1000 μw/cm^2, 650 mμ maximum emission) and protochlorophyll present following a subsequent 60 min dark period.

	Protochlorophyll			
Time after exposure to light	27° F_1[1]	27° M11	16° F_1[1]	16° M11
Min	*μg/g fr wt*			
0	1.1	1.1	0.3	0
60	5.8	4.9	1.3	0.5
Protochlorophyll formed in 60 min				
M11/F_1 %		81		59

[1] (K4 x M11).

Table VI. *Accumulation of Chlorophyll* a *in M11 Seedlings Following Different Exposures to Intermittent Light at 16°*

Seedlings were grown in the dark at 16° until first leaf was approximately 5 cm long, then illuminated with an electronic flash (1 msec duration, 5600° K).

	Chlorophyll *a*			
Line	1 hr[1]	5 hr[2]	48 hr[3]	96 hr[3]
	μg/g fr wt			
F_1 (K4xM11)	2	11		
K4			48	74
M11	1	3	19	48

[1] One light flash.
[2] Six light flashes at 1 hr intervals.
[3] Light flash at 30 min intervals.

Table VII. *Effect of Temperature on Carotenoid Content of Dark Grown Seedlings*

Seedlings were grown in the dark until the first leaf was approximately 5 cm long. The tips of leaves were discarded before assay. Values are the mean of 3 experiments.

Line	Carotenoid 27°	16°
	μg/g fr wt	
F_1 (K4 × M11)	66	43
M11	68	21

In addition to lower protochlorophyll synthesis, the level of carotenoids was also lower at 16°. At 27° there was an equivalent concentration of carotenoids in dark grown seedlings of M11 and the F_1, but at 16° the mutant had half the concentration of carotenoids present in the F_1, (table VII). One feature of the mutant seedlings was the high carotenoid content of the tips of leaves etiolated at 16°. This was consistent with the observation of chlorophyll in these regions of material grown at 16° under low light.

Table VIII. *Photosynthesis at 16°*

Seedlings were greened by exposure to intermittent light followed by continuous low intensity light Photosynthetic rates at 2350 ft-c were measured on individual leaves.

Line	Chlorophyll $a + b$	Photosynthesis	
	μg/g fr wt	*mg CO_2/dm²/hr*	*mg CO_2/mg chl/hr*
K4	1939	8.4	3.4
M11	1404	4.3	2.8
M11/K4 %	72	51	82

Photosynthesis and Translocation. Rates of photosynthesis at 16° were determined for single leaves of seedlings of M11 and K4, in which chlorophyll was accumulated by a combination of intermittent and low light treatments (see Methods). The photosynthetic rate of the mutant was 50 % that of K4 on the basis of leaf area, 80 % on the basis of chlorophyll content (table VIII). Distribution of ^{14}C-labeled assimilates from the same leaves was similar in M11 and K4 and ^{14}C activity was distributed to the crown and root systems. These results indicate that the chloroplasts of the mutant, when permitted to develop under favorable light conditions at 16°, functioned normally in photosynthesis, and the carbon assimilates were redistributed in a regular manner throughout the plant.

Discussion

The inability of seedlings of the mutant (M11) to accumulate chlorophyll at temperatures below 17° under natural light conditions represents an extreme degree of temperature sensitivity in maize, in which the lower temperature threshold for chlorophyll formation has been increased by about 4 to 5°. At higher temperatures, and particularly above 20°, although the rate of greening in M11 tends to be slower, the seedlings are normal in appearance and development.

In this respect low temperature-sensitive mutants differ from the more common chlorophyll-deficient mutants (albinos) which express at all temperatures, and are invariably lethal when homozygous.

Low temperature sensitivity as expressed in M11 is fairly specific, affecting particularly the accumulation of chlorophyll and other pigments (carotenoids) associated with greening. Development in all other respects appears normal, and the early growth rate based on seed reserves was equal to and often superior to many other inbred lines which were able to synthesize chlorophyll at low temperature.

The pattern of segregation in controlled crosses suggests that the capacity to accumulate chlorophyll at low temperature is under the control of at least 3 genes. Gene mutations leading to chlorophyll deficiency are common in maize (4) and in the case of temperature sensitivity, probably represent a qualitative or quantitative change in the expression of the gene at low temperature. The temperature sensitivity of M11, however, may represent the true ancestoral condition, and the genes which restore chlorophyll formation at low temperature may well have evolved through natural selection along with the domestication of maize and its cultivation further north from its original habitat in the tropics. The widespread occurrence and variable expression of the condition in inbred lines presumably results from the loss of restorer genes during the process of inbreeding.

The primary site of the low temperature sensitivity in M11 appears to be located near the shoot apex where basal meristems of the leaves are developing and undergoing rapid cell division. Experiments with seedlings grown with a large temperature differential between various combinations of root, apex and shoot, indicate that the pattern of greening in the developing leaf is a reflection of the temperature at the shoot apex, and not of the leaf itself. This is also evident from the rapid greening of M11 seedlings in the light at low temperature following etiolation at 27°. Chlorophyll formed at low temperature as a result of either method of pre-treatment is apparently normal and is stable in the light.

This evidence suggests that the lack of chlorophyll in M11 at 16° is a consequence of a lesion affecting a relatively early stage of either chloroplast development or function, and thus indirectly the synthesis of the various chloroplast pigments.

The reduced rate of protochlorophyll synthesis and the lower level of carotenoids in dark grown seedlings of M11 at 16° support the view that the etioplasts (9) do not function normally at low temperature. Supplying delta-aminolevulinic acid under these conditions resulted in the accumulation of

protochlorophyll which indicated that lack of chlorophyll accumulation was not due to a block in chlorophyll biosynthesis subsequent to delta-aminolevulinic acid formation.

Despite this evidence for the impaired function of etioplasts and the reduced rate of pigment synthesis, seedlings of M11 are capable of forming chlorophyll at low temperature if maintained in the dark and exposed to an intermittent light flash. Plants greened in this way are able to photosynthesize actively and are capable of an independent existence in low light at 16°. The lack of chlorophyll accumulation at 16° under normal light conditions does not, therefore, resemble the *Chlorella* mutants described by Davies (5). In these mutants the loss of photosynthetic capacity resulted in the photodestruction of chlorophyll in the light unless glucose was supplied as a carbon source. A similar treatment applied to M11 at low temperature had no effect on chlorophyll accumulation in the light.

It would appear from these results that the absence of chlorophyll in M11 seedlings grown below 17° in the light, is due to the impaired development and functioning of chloroplasts at low temperature, and an associated increased sensitivity to photo-oxidation. In M11 the severity of photo-oxidation at low light intensity was equivalent to that reported by McWilliam and Naylor (11) in hybrid maize at 16° in high light intensity. This increased sensitivity in M11 may be due to the lower level of carotenoids and slower rate of chlorophyll synthesis. It may also indicate that the plastids of the mutant seedlings are sensitive to photodestruction as is suggested by the lack of recovery of light grown leaves of M11 seedlings when transferred from 16° to 27°.

The detailed structure of the plastids of M11 is the subject of a further communication.

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