Plant Physiol. (1969) 44. 567–583

Studies on a Maize Mutant Sensitive to Low Temperature
II. Chloroplast Structure, Development, and Physiology

Adele Millerd, D. J. Goodchild, and D. Spencer
Division of Plant Industry, CSIRO, Canberra, A.C.T., Australia

Received December 10, 1968.

Abstract. In the *Zea mays* L. mutant M11 grown in the dark at 15°, the ultrastructure of the etioplast is abnormal. The pigment content of the etioplasts is reduced but the *in vivo* absorption characteristics suggest that the normal protochlorophyll(ide)-holochrome is present. The lowered synthetic ability of the etioplasts is not primarily due to a reduced complement of plastid ribosomes. The plastids of mutant M11 grown in the light at 15° contain little pigment, are markedly deficient in ribosomes and their ultrastructure is abnormal. In mutant M11 grown at 15°, an extreme sensitivity of the plastid membranes to light was observed.

In the *Zea mays* L. mutant inbred (M11) there is essentially no chlorophyll accumulation at or below 17°. It has been shown that the primary site of this low temperature sensitivity in mutant M11 is the basal meristem, the area of cell division and early cell expansion (9). This paper examines the nature of the aberrant plastids formed at low temperature. The behavior of mutant M11 has been contrasted with that of the F_1 hybrid (K4 × M11) which has a normal temperature response. Comparisons have been made of material grown at high temperature, 27°, and low temperature, 15°.

Materials and Methods

Plant Material. The temperature-sensitive mutant line (M11) of maize (*Zea mays* L.) and the F_1 hybrid (K4 × M11) used in these experiments have been described (9).

Seedlings were grown in the dark or in artificially lit cabinets under low intensity illumination (300 ft-c) as previously described (9). Plants were grown for 7 days at 27°, or for 18 or 19 days at 15°. Light-grown plants were harvested when the second leaf was well developed and the third just emerging. In dark-grown material, the first leaf had extended approximately 2 cm beyond the ruptured tip of the coleoptile at sampling time.

In all experiments the first leaf only has been used. When M11 is grown at 15° under low light, the tips of the leaves are green. This green portion represents cells present in the embryo and laid down in the previous generation when the seed was formed under temperature conditions normal for growth of maize (9). Thus, when plants grown at 15° were used, the tips of leaves from both mutant M11 and the F_1 hybrid were discarded.

Development of Plastids in Intermittent Light. Intermittent light from a Braun EF 300 electronic flash unit activated once every 30 min was used. The flash duration was 1 msec with a color temperature of 5600°K at an output of 135 watt sec. The light source was mounted approximately 4 inches above the leaf tips.

Extraction and Estimation of Plastid Pigments. Material was extracted with 85 % acetone and chlorophyll *a* and *b*, protochlorophyll(ide) and carotenoids were estimated as described previously (9).

Absorption Characteristics of Protochlorophyll(ide) and Chlorophyll(ide) a in Vivo. The *in vivo* absorption characteristics of protochlorophyll(ide) and chlorophyll(ide) *a* formed after exposure to red light [30 sec, 3 cm from a red fluorescent tube (Philips TL 20W/15), maximum emission 650 nM, light intensity 350 $\mu w/cm^2$ as measured by a Zeiss thermopile], were determined with a Cary Model 14R spectrophotometer fitted with a Cary Model 1462 scattered transmission attachment. The cuvette assembly of Boardman and Highkin (2) was used: except that the leaves were clamped tightly between 2 perspex windows. A diluted suspension of milk served as an appropriate scattering blank in the reference cuvette. In material grown at 27°, the measuring light was passed through 3 leaves and spectra examined at 20°. With material grown at 15°, it was necessary to use more material (6 leaves of F_1 hybrid and 9 leaves of mutant M11) and the spectra were examined at 77°K.

Isolation and Analysis of Ribosomes. Leaf material, 4 to 5 g, was rapidly harvested, weighed, chilled and chopped finely with scissors. The material was ground in a mortar with 2 vol tris-Mg-SH medium (tris buffer, 0.02 M, pH 7.8; $MgCl_2$, 0.02 M, mercaptoethanol 1 mM) containing 4 % Triton X-100. The brei was strained through 2 layers of Miracloth and the residue washed with 1 ml tris-Mg-SH medium containing 4 % Triton X-100. This cell-free filtrate was centrifuged at 20,000*g* for 20 min. The supernatant was then centrifuged at 144,000*g* for 90 min. The resulting supernatant was discarded,

the tubes drained thoroughly and the pellet suspended slowly (1 hr) in 1 ml tris-Mg-SH medium. The suspension which was primarily ribosomes, was clarified by centrifugation (20,000*g* for 10 min).

Ultracentrifugal analyses were carried out in a Spinco Model E centrifuge with schlieren optics and standard 12 mm cell. Samples were run in the above tris-Mg-SH medium at a nucleic acid concentration of 5 mg/ml. Photographs were taken at a bar angle of 40° approximately 16 min after reaching speed (42,040 r.p.m.).

Examination of Ultrastructure of Plastids. Prior to fixation, plants were cut off at ground level in the artificially lit cabinets or in the dark, wrapped in foil, and taken to the laboratory for sampling and fixation in the light. Several leaf pieces, approximately 1 mm², were cut from the center of the first leaf, fixed in 3 % glutaraldehyde (purified by vacuum distillation) in 0.025 M or 0.1 M Na phosphate buffer at pH 7.2 for 1.5 hr at 20°, washed in 4 changes of buffer for a total of 1 hr and then post-fixed in 2 % osmium tetroxide in 0.025 M phosphate buffer at pH 7.2 for 2 hr at 20°. Dark-grown plants harvested in the dark and exposed only to green light until the end of the osmium fixation showed no ultrastructural differences from those fixed in the light. After washing in buffer and dehydration in ethanol and propylene oxide, the leaf pieces were embedded in an araldite-epon mixture (10). The resin was vacuum infiltrated at 85° and polymerized at the same temperature for 24 hr before sectioning with an LKB or Reichert ultra-microtome. Sections were stained with saturated uranyl acetate in 50 % ethanol for 1 hr followed by Fiske's (4) lead stain before examination in a Philips EM 200 electron microscope.

Results

Pigment Content of F_1 Hybrid and Mutant M11 Under Various Growth Conditions. The pigment content of seedlings of both the F_1 hybrid and M11 was dependent on growth conditions. The results in table I are the mean of 5 experiments. The absolute values varied (approximately ± 10 %) between experiments but the relationship of the 2 lines was constant. At 27°, in light or dark, the 2 lines behave similarly. At 15° in both light and dark there is a marked difference and the pigment content of mutant M11 is characteristically lower than that of the F_1 hybrid with a particularly marked reduction in chlorophyll content in the light.

In vivo *Absorption Characteristics of Protochlorophyll(ide) and Chlorophyll(ide) a in F_1 Hybrid and Mutant M11.* The *in vivo* absorption maxima of protochlorophyll(ide) and chlorophyll(ide) *a* in the F_1 hybrid are identical to their counterparts in M11 at high and low temperature. Because of the low pigment content of seedlings grown at 15° absorption peaks in this material were measured at 77°K. This resulted in the protochlorophyll(ide) 650 nM and 636 nM absorption maxima shifting to 651 nM and 637 nM respectively and the chlorophyll(ide) *a* absorption maxima shifting from 683 nM to 681.5 nM. In both maize lines, grown at 27° or 15°, some untransformed protochlorophyll(ide) absorbing at approximately 632 nM remained after exposure to red light. In the F_1 hybrid and mutant M11 grown in the dark at 15° there were no differences in the relative proportions of protochlorophyll(ide) 650 and the minor component absorbing at 636 or 637 nM and the amount of transformation after red light treatment was similar in both.

Table I. *Effect of Growth Conditions on Pigment Content of F_1 Hybrid and M11*

Seedlings were grown in the light (300 ft-c) at 27° or 15° until appearance of the third leaf. Material grown in the dark was the same age as the appropriate light-grown material (7 days at 27°, 19–20 days at 15°). Values are means of 5 experiments.

	27°		15°	
	F_1 (K4×M11)	M11	F_1 (K4×M11)	M11
	μg/g fresh wt			
Dark				
Protochlorophyll(ide)	5.8	4.5	3.1	1.3
Carotenoid	64	70	54	23
Light				
Chlorophyll *a* + *b*	1814	1550	880	12

Ribosome Content of Plastids of F_1 Hybrid and Mutant M11. The relative amounts of chloroplast (70S) and cytoplasmic (80S) ribosomes were examined to determine if the reduced capacity for pigment synthesis was associated with a marked reduction in the ribosome population of the mutant's plastids.

In plants grown at 27° (Fig. 1), the ratios of 70S:80S ribosomes were similar in the F_1 hybrid and mutant M11 in the dark (Fig. 1a, 1b) and were markedly increased in both lines in the light (Fig. 1c, 1d). When plants are grown at 15° in the dark, (Fig. 2) the ratio of 70S:80S ribosomes is similar for the F_1 hybrid (Fig. 2a) and mutant M11 (Fig. 2b). In the light (300 ft-c) at 15°, there is a marked reduction in the 70S component (Fig. 2d) as compared with that present in the F_1 hybrid (Fig. 2c).

Ultrastructure of Plastids of F_1 Hybrid and Mutant M11. Examination of the ultrastructure of plastids of mutant M11 grown at 27°, shows (Fig. 3a) that both the etioplast (7) and the mature chloroplast (Fig. 3b) have the structure classically associated with mesophyll plastids of *Zea mays* L. (5, 12). The ultrastructure of the chloroplast is identical in material grown under 300 or 2000 ft-c of continuous light, or 2000 ft-c for 16 hr and 8 hr dark. In both the F_1 hybrid and mutant M11 plants grown at 27° the ultrastructure of both bundle-sheath and mesophyll plastids is normal and in agreement

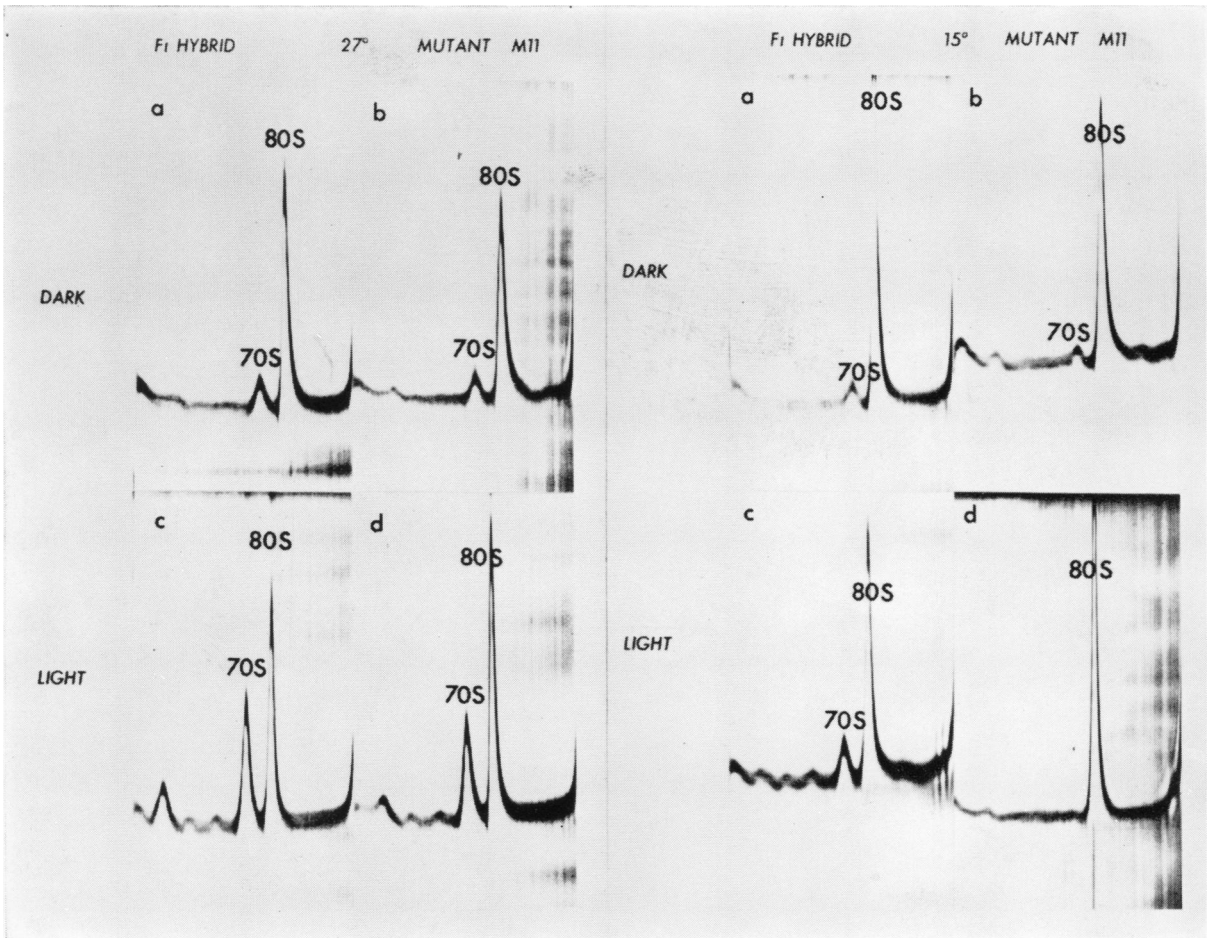

FIG. 1. (left) Analytical ultracentrifuge patterns of ribosomes from extracts of whole leaves from plants grown at 27°. Seedlings grown in the dark (a) F_1 hybrid (b) M11. Seedlings grown in 300 ft-c continuous light (c) F_1 hybrid (d) M11. Samples were run in tris 0.02 M, pH 7.8; $MgCl_2$, 0.02 M, mercaptoethanol 1 mM, at a nucleic acid concentration of 5 mg/ml. Photographs were taken approximately 16 min after reaching 42,040 rpm using schlieren optics and a bar angle of 40°.

FIG. 2. (right) Analytical ultracentrifuge patterns of ribosomes from extracts of whole leaves from plants grown at 15°. Seedlings grown in the dark (a) F_1 hybrid (b) M11. Seedlings grown in 300 ft-c continuous light (c) F_1 hybrid (d) mutant M11. Experimental details as described in Fig. 1.

Insert

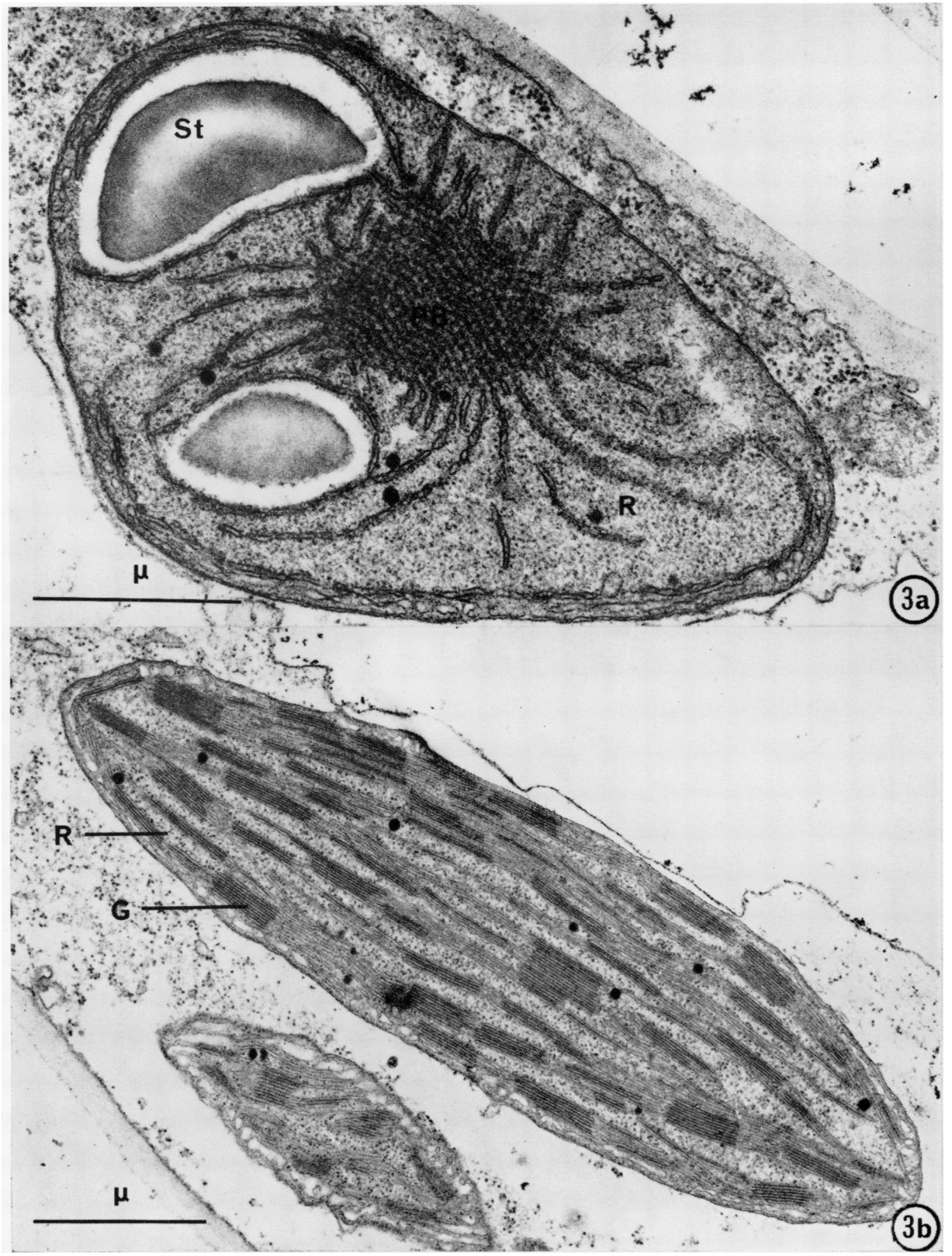

FIG. 3. Structure of mutant M11 plastids in plants grown at 27°.

FIG. 3a. Etioplast from dark grown plants showing *para*-crystalline prolamellar body (PB), starch grains (St) and ribosomes (R).

FIG. 3b. Chloroplasts from plants grown at 300 ft-c showing regular grana (G) and ribosomes (R).

Insert

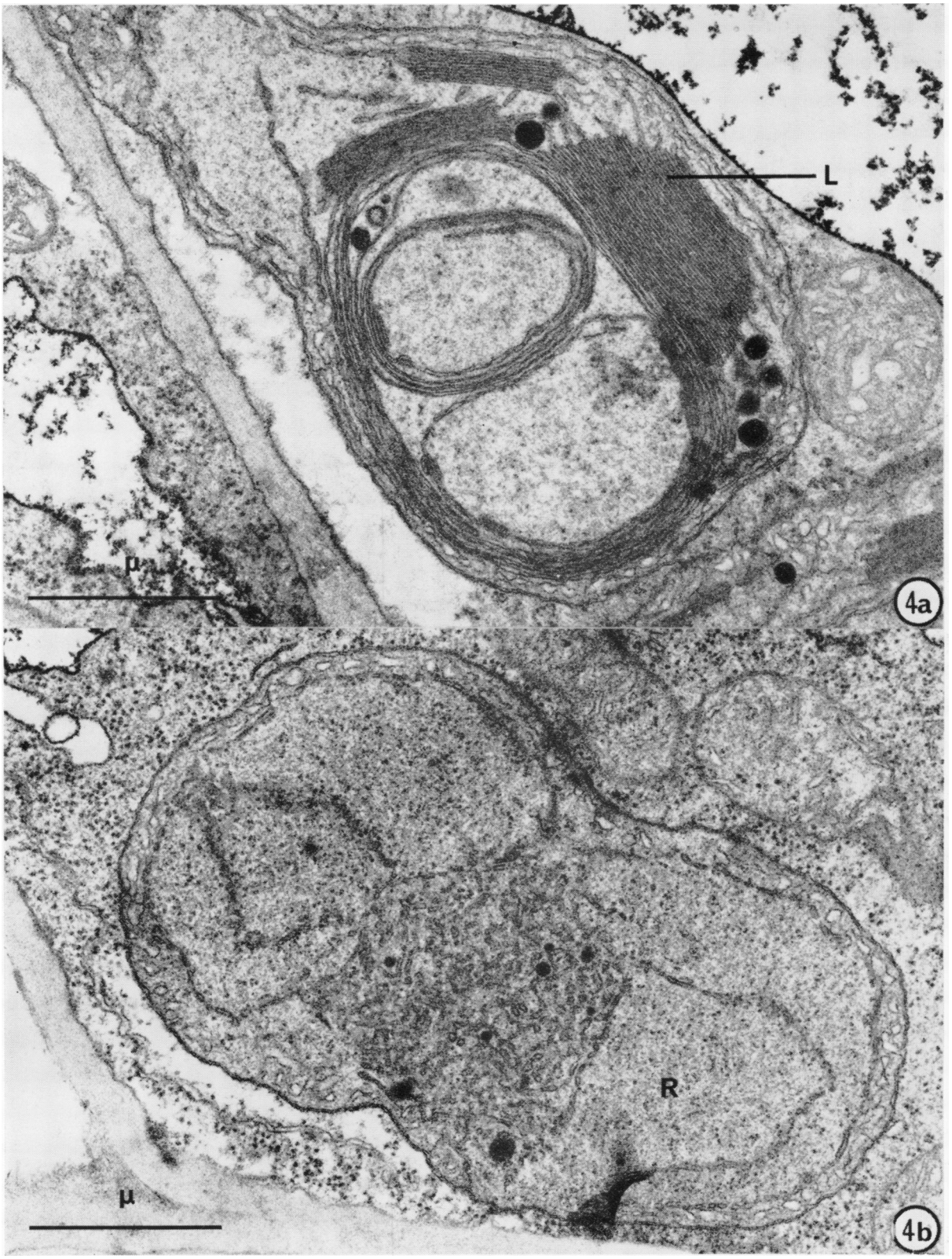

FIG. 4. Structure of plastids in F_1 hybrid and mutant M11 plants grown at 15°.

FIG. 4a. F_1 hybrid chloroplast from plants grown at 2000 ft-c showing lamellae arranged as 1 massive stack (L) and accompanied by whorls and smaller stacks.

FIG. 4b. Mutant M11 etioplast showing the maximum internal membrane order observed and ribosomes (R). Note absence of any *para*-crystalline prolamellar bodies.

Insert

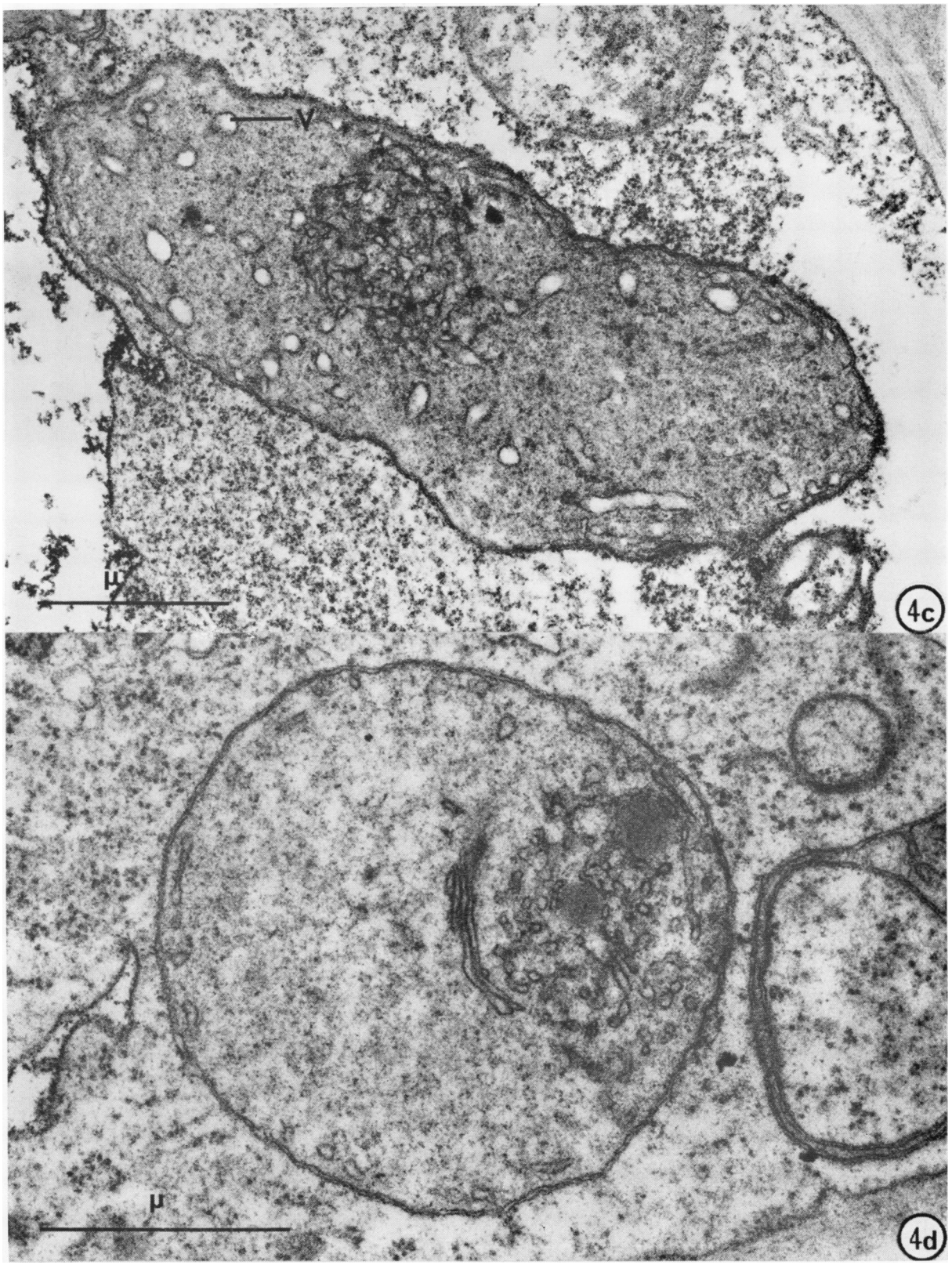

FIG. 4c. Mutant M11 etioplast showing the more characteristic arrangement of internal membranes with vesiculation (V).

FIG. 4d. Mutant M11 plastid from plants grown at 300 ft-c showing few internal membranes and an absence of clearly defined chloroplast ribosome profiles.

Insert

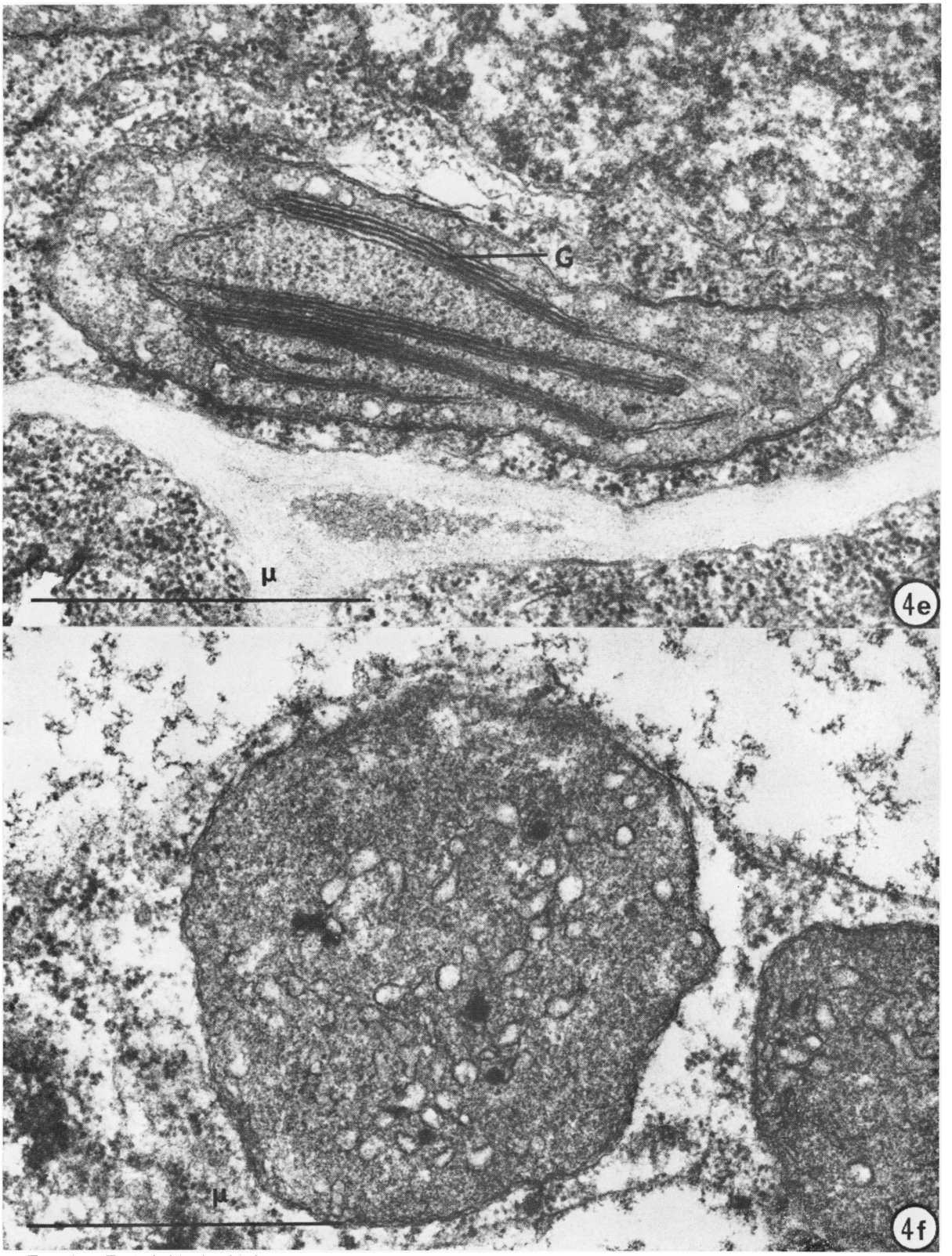

FIG. 4e. F_1 hybrid plastid from the meristematic region of a leaf grown at 300 ft-c showing rudimentary grana formation (G).

FIG. 4f. Mutant M11 plastid from the meristematic region of a leaf grown at 300 ft-c showing vesiculation and no grana.

Insert

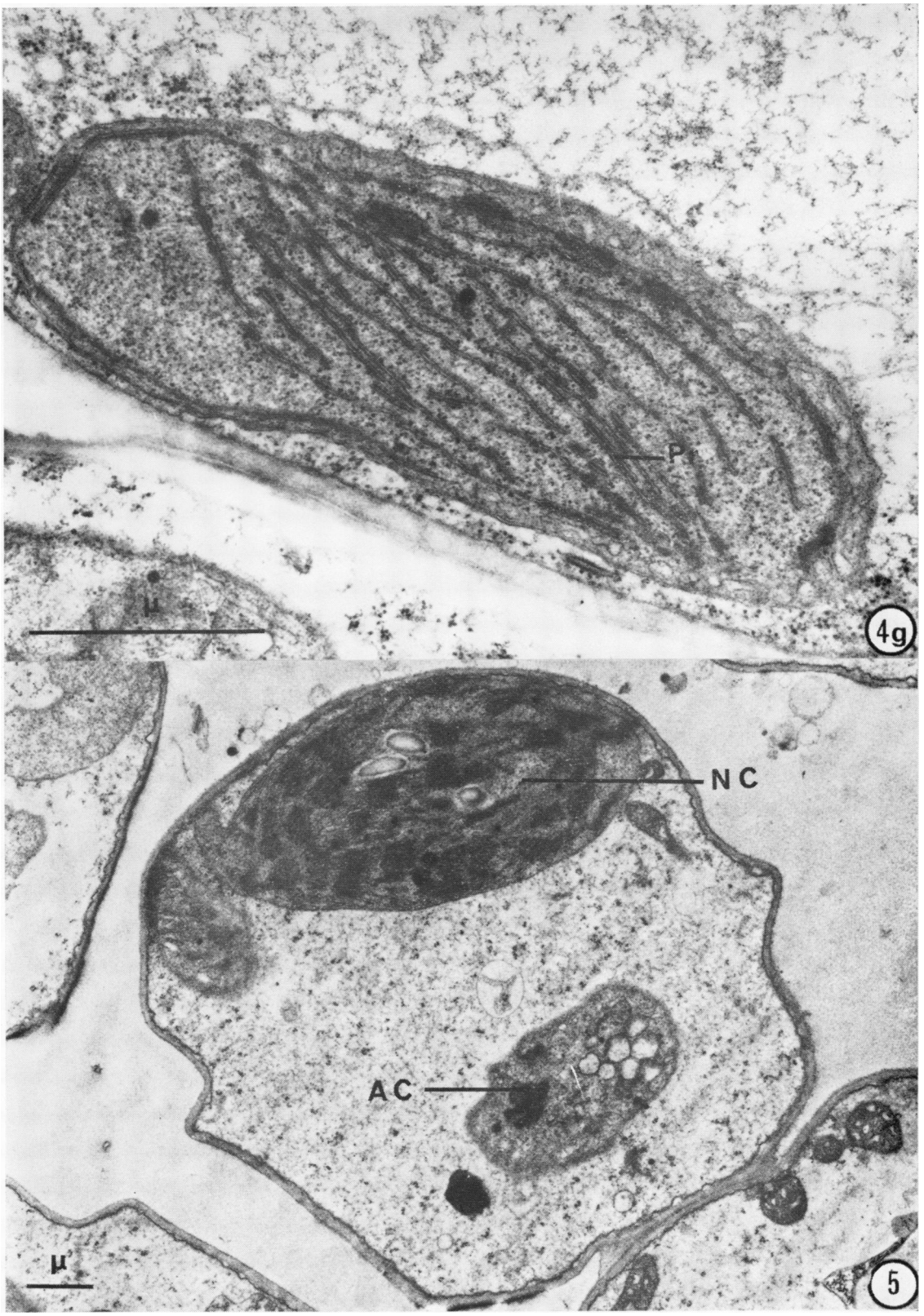

FIG. 4g. Mutant M11 plastid from plants grown in intermittent light showing internal lamellae with few sites of pairing (Pr).

FIG. 5. Chloroplasts developed at 15° in mutant M11 seedlings grown at 27° and then transferred to 15°. A comparatively normal chloroplast (NC) and an abnormal chloroplast (AC) in a single cell.

Insert

with ultracentrifugal analyses, the plastids contain a large complement of ribosomes.

When the F_1 hybrid is grown at 15° in the dark, the etioplast is indistinguishable from that observed in plants grown at 27° and is similar to that described by Jacobson *et al.* (6).

When the F_1 hybrid is grown at 15° in the light, more variation in chloroplast structure is observed. In contrast with plants grown at 27° the ultrastructure of the chloroplast is dependent on light intensity. At 300 ft-c, chloroplast structure is indistinguishable from material grown at high temperature (Fig. 3b). At 15°, under 2000 ft-c continuous light, many chloroplasts contained essentially one granal stack (Fig. 4a).

When mutant M11 is grown in the dark at 15°, the elements of the prolamellar body of the etioplast are not organized into the characteristic para-crystalline array. Fig. 4b shows the most highly organized prolamellar body observed in this material. Most prolamellar bodies had much less organization (Fig. 4c). The less organized state is not due to slower development, since with increasing age of plants (up to 25 days), the degree of organization was found to decrease.

The inbred K4, the female parent of the F_1 hybrid, when grown at 15° in the light, is a small seedling but the chlorophyll content is *ca.* 60 % that of the F_1 hybrid (9). When the etioplasts of K4 grown at 15° were examined, the characteristic para-crystalline array was lacking in the prolamellar body but not to such a marked degree as in mutant M11.

The ultrastructure of the chloroplasts of mutant M11 grown at 15° in the light (300 ft-c) was typically that shown in Fig. 4d. Few internal membranes remained and no clearly distinguishable chloroplast ribosome profiles are visible, compared with dark grown material (Fig. 4b and 4c). The plastids of the vascular bundle cells were also structurally abnormal but in some plastids ribosomes were visible. This type of ultrastructure observed in the mutant M11 is very different from the 'bleached' plastids observed by McWilliam and Naylor (8). They showed that photo-oxidation of pigments was not necessarily accompanied by the destruction of chloroplast membranes and ribosomes. In their experiments etiolated maize was transferred to high intensity light (4,500 ft-c) at low temperature (16°) but chlorophyll did not accumulate. We found that in such plants, even after exposure to light (4,500 ft-c) for 96 hr vestiges of prolamellar bodies were still present, a network of membranes and tubules existed and ribosomes were abundant. When these plants are transferred to a higher temperature chlorophyll rapidly accumulates and the leaf appears normal, in marked contrast with the mutant M11.

During the early stages of cell development in mutant M11, temperature is critical (9). We have examined the ultrastructure of proplastids from the meristematic region of plants grown in light at 15°. In the developing plastids of the F_1 hybrid (Fig. 4e), immature granal stacks are readily seen. In contrast, in mutant M11 such structures are lacking (Fig. 4f).

Development of Plastids in Intermittent Light. In intermittent high intensity light chlorophyll will accumulate in M11 at 15° (9), and under these conditions photooxidation is minimized. The hybrid and mutant M11 were compared under these conditions and, as is shown in table II, chlorophyll *a* accumulated but, in acetone extracts, no chlorophyll *b* could be detected by fluorescence spectroscopy at room temperature. Chlorophyll accumulation was, as would be expected, much more rapid at 27°, and at 15°, the chlorophyll content of the hybrid was consistently higher than that of the mutant.

Table II. *Accumulation of Chlorophyll a Following Exposure to Intermittent Light*

Seedlings were grown in the dark at 15° until the first leaf was approximately 5 cm long, then illuminated with an electron flash (1 msec duration, 5,600° K) every 30 min. First leaves, with tips removed, were used for pigment assay.

		Duration of intermittent light hr			
Line	Temp	8	24	48	72
		Chlorophyll *a*			
		μg/g fresh wt			
F_1 (K4 × M11)	27	97			
	15		13	56	124
M11	27	84			
	15		6	27	55

Ultrastructural changes in the plastids were also examined. At 27°, after 8 hr (17 flashes) considerable membrane development had occurred in both hybrid and mutant. At 15°, the pattern of development in the hybrid was similar but slower than at 27°. In the mutant at 15°, wide variation in plastid structure was observed. Many plastids had disintegrated and even in those showing maximal structural development (Fig. 4g) grana were never seen although some lamellae did pair to form partitions. In the mutant at 15°, it was not possible to correlate chlorophyll content and structure, since the pigment assays represented the average of all cells and such large variations were observed between individual plastids.

Transfer of Mutant M11 Seedlings From 27° to 15°. Young seedlings of mutant M11 were grown at 27°, then transferred to 15°. The new leaf tissue which appeared was very pale but some chlorophyll was evident in the region of the vascular bundles. Electron microscopy of the chloroplasts of this region revealed that the plastids in cells of the bundle sheath were essentially normal, but the population of nearby mesophyll cells included abnormal and normal plas-

tids (Fig. 5). Normal chloroplasts were not observed in mesophyll cells distant from the vascular bundles.

These observations suggested the transport, from the green portion of the leaf, of a substance or substances which promoted the development of the normal chloroplasts in M11 at 15°. A large number of small molecular weight substances have been supplied to M11 growing on agar at 15°, but no significant increases in chlorophyll accumulation were observed.

Discussion

When maize mutant M11 is grown at 27°, either in light or dark, the pigment content and the ultrastructure of the plastids are comparable with those of the F_1 hybrid (K4 × M11).

When mutant M11 is grown at 15°, in the light or dark, its plastids are abnormal. In the dark the elements of the prolamellar body are not organized into the characteristic *para*-crystalline array (Fig. 4b, 4c). Mutant M11, then, differs from the carotenoid-deficient maize mutant described by Robertson *et al.* (14) where plastid development in the dark-grown mutant is normal.

The pigment content of etioplasts of mutant M11 is low at 15° (table I) but the absorption characteristics of chlorophyll(ide) *a* formed *in vivo* suggest that the normal protochlorophyll(ide)-holochrome is present. The relative proportions of protochlorophyll(ide) with an absorption maximum of 650 nM (P650) and protochlorophyll(ide) with an absorption maximum of 636 nM (P636) are similar in the F_1 hybrid and mutant M11. This observation contrasts with the suggestion (3) that the P650 form is present only in association with a prolamellar body.

When mutant M11 is grown in the dark at 15° the rate of pigment synthesis is low (table I and reference 9) but this is not primarily a reflection of a reduced complement of plastid ribosomes since the ratio of 70S (plastid) to 80S (cytoplasmic) ribosomes in such material (Fig. 2b) approximates that in the F_1 hybrid (Fig. 2a) grown under the same conditions. These observations are supported by electron micrographs (Fig. 4b, 4c).

Maize is a tropical plant and even in the F_1 hybrid grown at 15°, under certain conditions, the plastids may differ from those in plants grown at 27°. At 15°, the ultrastructure of the chloroplast is dependent on light intensity. Under illumination of 300 ft-c, the structure is normal but under 2000 ft-c abnormal granal stacks were observed (Fig. 4a). In addition, in the light at 15°, there is a relatively lower rate of plastid ribosome synthesis since the ratio of 70S:80S ribosomes is much higher at 27° (Fig. 1c) than at 15° (Fig. 2c).

The plastids of mutant M11 grown in the light at 15° have a very low pigment content (table I), their ultrastructure is abnormal (Fig. 4d) and they are markedly deficient in ribosomes (Fig. 2d, 4d). The deterioration of the internal structure of the plastids and the disappearance of 70S ribosomes in mutant M11 in the light is quite similar to that described by Bartels *et al.* (1) in barley germinated in the presence of 3-amino-1,2,4-triazole (AT). The ultrastructure of the barley etioplasts was considered normal but the plastids of the light-grown, AT-treated plants had poor internal structure and lacked 70S ribosomes.

When mutant M11 is grown at 15° the plastid membranes seem particularly sensitive to light (Fig. 4d). This is supported by 2 further observations. Firstly, using intermittent high intensity light, chlorophyll will accumulate in mutant M11 at 15° (9). The striking difference between the response of mutant M11 and the F_1 hybrid under these conditions is in membrane development. Though many plastids lacked internal structure, even in those which showed maximal structural development, normal membrane arrangements were rarely seen (Fig. 4g). Secondly, the meristematic region of mutant M11 is the primary site of low temperature sensitivity (9). At 15° in the light, vestiges of granal formation are present in the proplastids of the meristematic region of the F_1 hybrid (Fig. 4e) but these are not evident in mutant M11 (Fig. 4f).

Certain cold-sensitive *E. coli* mutants have been shown to grow normally at low temperature when minimal medium is supplemented with particular amino acids or vitamins (11). It has not been possible to increase significantly chlorophyll accumulation at 15° by growing mutant M11 on agar supplemented with a wide variety of small molecular weight substances.

However there are indications that a transportable substance is involved. When mutant M11 seedlings were grown at high temperature and then transferred to low temperature, the new tissue had normal chloroplasts in the cells of the bundle sheath and neighboring mesophyll cells (Fig. 5) contained some normal plastids. This result suggests transport from the green leaf of a substance or substances which promoted, at 15°, the normal chloroplast development in mutant M11.

Acknowledgments

We are indebted to Dr. N. K. Boardman for carrying out the *in vivo* pigment studies and to Mr. S. W. Thorne for fluorescence measurements. The technical assistance given by Miss Elizabeth Smith and Miss Margaret Paulin is also gratefully acknowledged.

Literature Cited

1. Bartels, P. G., K. Matsuda, A. Siegel, and T. E. Weier. 1967. Chloroplastic ribosome formation: inhibition by 3-amino-1,2,4-triazole. Plant Physiol. 42: 736–41.

2. BOARDMAN, N. K. AND H. R. HIGHKIN. 1966. Studies on a barley mutant lacking chlorophyll *b*. I. Photochemical activity of isolated chloroplasts. Biochim. Biophys. Acta 126: 189–99.
3. BUTLER, W. L. AND W. R. BRIGGS. 1966. The relation between structure and pigments during the first stages of proplastid greening. Biochim. Biophys. Acta 112: 45–53.
4. FISKE, S. 1966. An adaptation of Reynolds' lead citrate stain for high resolution autoradiography. J. Microscopie 5: 355–60.
5. HODGE, A. J., J. D. MCLEAN, AND F. V. MERCER. 1955. Ultrastructure of the lamellae and grana in the chloroplasts of *Zea mays* L. J. Biophys. Biochem. Cytol. 1: 605–13.
6. JACOBSON, A. B., H. SWIFT, AND L. BOGORAD. 1963. Cytochemical studies concerning the occurrence and distribution of RNA in plastids of *Zea mays*. J. Cell Biol. 17: 557–70.
7. KIRK, J. T. O. AND R. A. E. TILNEY-BASSETT. 1967. The plastids. W. H. Freeman and Company, London and San Francisco, California.
8. MCWILLIAM, J. R. AND A. W. NAYLOR. 1967. Temperature and plant adaptation. I. Interaction of temperature and light in the synthesis of chlorophyll in corn. Plant Physiol. 42: 1711–15.
9. MILLERD, A. AND J. R. MCWILLIAMS. 1969. Studies on a maize mutant sensitive to low temperature. I. Influence of temperature and light on the production of chloroplast pigments. Plant Physiol. 43: 1967–72.
10. MOLLENHAUER, H. H. 1964. Plastic embedding mixtures for use in electron microscopy. Stain Technol. 39: 111–14.
11. O'DONOVAN, G. A., C. L. KEARNEY, AND J. L. INGRAHAM. 1965. Mutants of *Escherichia coli* with high minimal temperatures of growth. J. Bacteriol. 90: 611–16.
12. PAOLILLO, D. J. AND R. H. FALK. 1966. The ultrastructure of grana in mesophyll plastids of *Zea mays*. Am. J. Botany 53: 173–80.
13. ROBERTSON, D. S., M. D. BACHMANN, AND I. C. ANDERSON. 1966. Biochemical and plastid alterations in allelic mutants of the W_3 locus in maize. Genetics 54: 357–58, Abstr.