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Plastid Development in Albescent Maize[1]

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Abstract. Plastid development in albescent (al/al) and wild-type (+/al) strains of *Zea mays* has been studied in the electron microscope. Etiolated seedlings of the mutant are severely deficient in colored carotenoid pigments and accumulate carotenoid precursors tentatively identified as phytoene and phytofluene. The fine structure of proplastids in etiolated wild-type and mutant leaves is similar with 1 notable exception. Osmiophilic bodies found in the wild-type were lacking in all sections of albescent proplastids examined suggesting that these structures may be storage centers for carotenoid pigments. Plastid pigments are destroyed, chlorophyll synthesizing potential is lost, and the ultrastructure of plastids is irreversibly altered when mutant seedlings are placed directly in high intensity light. However, synthesis of plastid pigments and development of the photosynthetic apparatus as seen in the electron microscope is normal, and indistinguishable from that in the wild-type, in seedlings of the albescent mutant preilluminated with low intensity light prior to high intensity illumination. During treatment in low intensity light carotenogenesis is initiated in the mutant and proceeds normally thereafter.

Pigment deficiency in algae and higher plants is a common consequence of gene mutation. More often than not, the block in pigment synthesis persists throughout the life of the organism. This is particularly evident in mutants with defects in carotenoid biosynthesis. For instance, albino and xantha mutants of sunflower described by Walles (20), albino mutants of maize described by Koski and Smith (11), Smith *et al.* (19), and Anderson and Robertson (1), and a *Chlamydomonas* mutant (17), are variously deficient in colored carotenoids and unable to correct their respective defects under any of the growth or culture conditions tested. The albescent mutant of maize (al/al) is an interesting carotenoid deficient strain (16) in that carotenoid synthesis is blocked in plants grown in darkness, but following a suitable light regime carotenoid pigment is produced in amounts comparable to that in the wild-type (+/al). In the present paper, chloroplast development in the albescent (al/al) and wild-type (+/al) strains of *Zea mays* is compared.

Materials and Methods

The seed stock of albescent (al/al) and wild-type (+/al) *Zea mays* (kindly supplied by Dr. W. D. Bell) has been described previously (16). Seeds were soaked in tap water for 3 hr and germinated in moist vermiculite. Seedlings were grown at 25° for 7 days in darkness, in low intensity light, or in high intensity light. Light was provided by fluorescent tubes (power groove, GE F72, CW) supplemented with incandescent filament lamps (75 watt). Low and high intensity light conditions furnished 1.5×10^3 and 3.5×10^4 ergs/cm^2 sec^{-1}, respectively, at plant height. Light intensity measurements were made with a YSI-Kettering thermistor radiometer. Low intensity light conditions were obtained by placing seedlings in a topless cardboard box covered with multiple layers of cheesecloth.

For electron microscopy, squares of tissue (*ca.* 1 mm × 1 mm) were removed approximately 1 cm from the tip of the second leaf at a position equidistant between the midrib and border. The tissue squares were fixed for 2 hrs in 3 % glutaraldehyde (v/v) buffered with 0.1 M potassium phosphate buffer, pH 6.8 and postfixed in 1 % osmium tetroxide for the same period. The samples were dehydrated in a graded series of alcohols and embedded in EPON (13). Thin sections cut with glass knives on a Porter-Blum ultramicrotome were placed on 300 mesh grids and stained for 10 min with 1 % uranyl acetate and lead citrate (15). Grids were examined in a Siemans Elmiskop I electron microscope.

Chloroplast pigments were extracted by grinding weighed leaves in 80 % (v/v) acetone with sand in a mortar and pestle. Extracts were clarified by filtration through asbestos. Chlorophyll-*a* and chlorophyll-*b* concentrations were estimated from the optical density (OD) of pigment in 80 % acetone at

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663 and 645 nm, respectively, by Arnon's method (2), using the specific absorption coefficients of MacKinney (14). For the determination of carotenoid pigment concentrations, 80 % acetone extracts were diluted with 2 volumes of 10 % NaCl and the pigments transferred to hexane. The hexane was dried *in vacuo*, the residue was saponified for 14 hrs in 30 % methanolic KOH at room temperature, and the unsaponified pigments were taken back into hexane (8). Crude xanthophylls were obtained by extraction of the resulting hexane solution with 92 % methanol. Carotenes (in hexane) and xanthophylls (in 92 % methanol) were estimated spectrophotometrically from the OD of pigment at 470 and 465 nm, respectively. Spectra and OD measurements were made on Beckman DB and DU spectrophotometers.

To determine the effect of mutation at the albescent locus on the metabolic pathway for chlorophyll synthesis, etiolated leaves from wild-type (+/al) and mutant (al/al) plants were excised and incubated in darkness at 25° for 12 hrs on 0.2 M sucrose solutions containing the chlorophyll precursor, 0.01 M Δ-aminolevulinic acid (ALA). Control leaves were incubated on solutions containing 0.2 M sucrose alone. Protochlorophyllide-*a* produced in leaves during incubations with Δ-aminolevulinic acid was extracted with 80 % acetone from ground leaves. The concentration of protochlorophyllide-*a* in the extracts was estimated from the OD at 623 nm ($E^{.1\%}_{1cm}$ = 34.9 in 80 % acetone (9).

Results

Etiolated Plants. The ultrastructural features of proplastids in mesophyll cells of wild-type (+/al) and albescent (al/al) *Zea mays* are shown in Fig. 1 and 2, respectively. Proplastids in plants of either genotype were round to slightly oval, 3 to 4 μ in diameter, and contained a large crystalline prolamellar body with tubular strands extending into the surrounding stroma. Ribosomes were present in the stroma and vesicles occurring singly or in clusters were located adjacent to the proplastid membrane. Starch grains were usually present in the stroma but sometimes interrupted the continuity of the prolamellar body. One difference between albescent and wild-type proplastids are observed consistently. Osmiophilic bodies found in the stroma or in discontinuous areas of the prolamellar body in the wild-type were not observed in proplastids of the albescent mutant.

Table I. *Pigmentation in Etiolated Albescent and Wild-type Seedlings*

Pigment concentrations are expressed on a gram-fresh weight basis. Each value is the average of 3 determinations.

Genotype	Protochloro-phyllide-*a*	Carotene	Xantho-phyll
	mg/g×*10*⁻³	*OD 470/g*	*OD 465/g*
1) (+/al)	8.1	3.2	5.0
2) (al/al)	8.4	0.2	0.3

Comparable quantities of protochlorophyllide-*a* were present in albescent and wild-type leaves (table I). Protochlorophyllide-*a* in the mutant was converted to chlorophyllide-*a* during exposure for 5 min to high intensity light. The carotenoid pigment concentration in dark-grown leaves of the 2 strains differed significantly. The quantity of pigment in crude "carotene" and crude "xanthophyll" fractions from albescent leaves was one-sixteenth and one-seventeenth of that found in the corresponding fractions obtained from wild-type leaves (table I). In place of colored carotenoid pigments, albescent seedlings contained ultraviolet light-absorbing carotenoid precursors. These were judged to be phytoene and phytofluene on the basis of their absorption maxima in hexane (following saponification) at 298, and 368, 348, and 331 nm, respectively (8). Based on OD measurements, the quantity of carotenoid precursors in the mutant was about one-tenth the quantity of carotenoid pigment present in the dark-grown wild-type strain.

Synthesis of protochlorophyllide-*a* from ALA was tested in albescent and wild-type leaves. Excised dark-grown leaves were placed on sucrose solutions containing 0.01 M ALA. During 12-hr incubations at 25° in darkness, albescent and wild-type leaves synthesized about 10 times the quantity of protochlorophyllide-*a* present initially (table II). Con-

Table II. *Pigment Synthesis in Detached Albescent and Wild-type Leaves*

Concentrations of pigment are expressed on a gram-fresh weight basis. Each value is the average of 3 determinations.

Genotype	Conditions	Protochlorophyllide-*a*	Chlorophyll (*a*+*b*)
		mg/g × *10*⁻²	*mg/g* × *10*⁻²
1. (+/al)	Dark, sucrose	0.8	...
2. (+/al)	Dark, sucrose, ALA	7.4	...
3. (al/al)	Dark, sucrose	0.8	...
4. (al/al)	Dark, sucrose, ALA	6.3	...
5. (+/al)	Light, sucrose	...	13.0
6. (al/al)	Light, sucrose	...	0

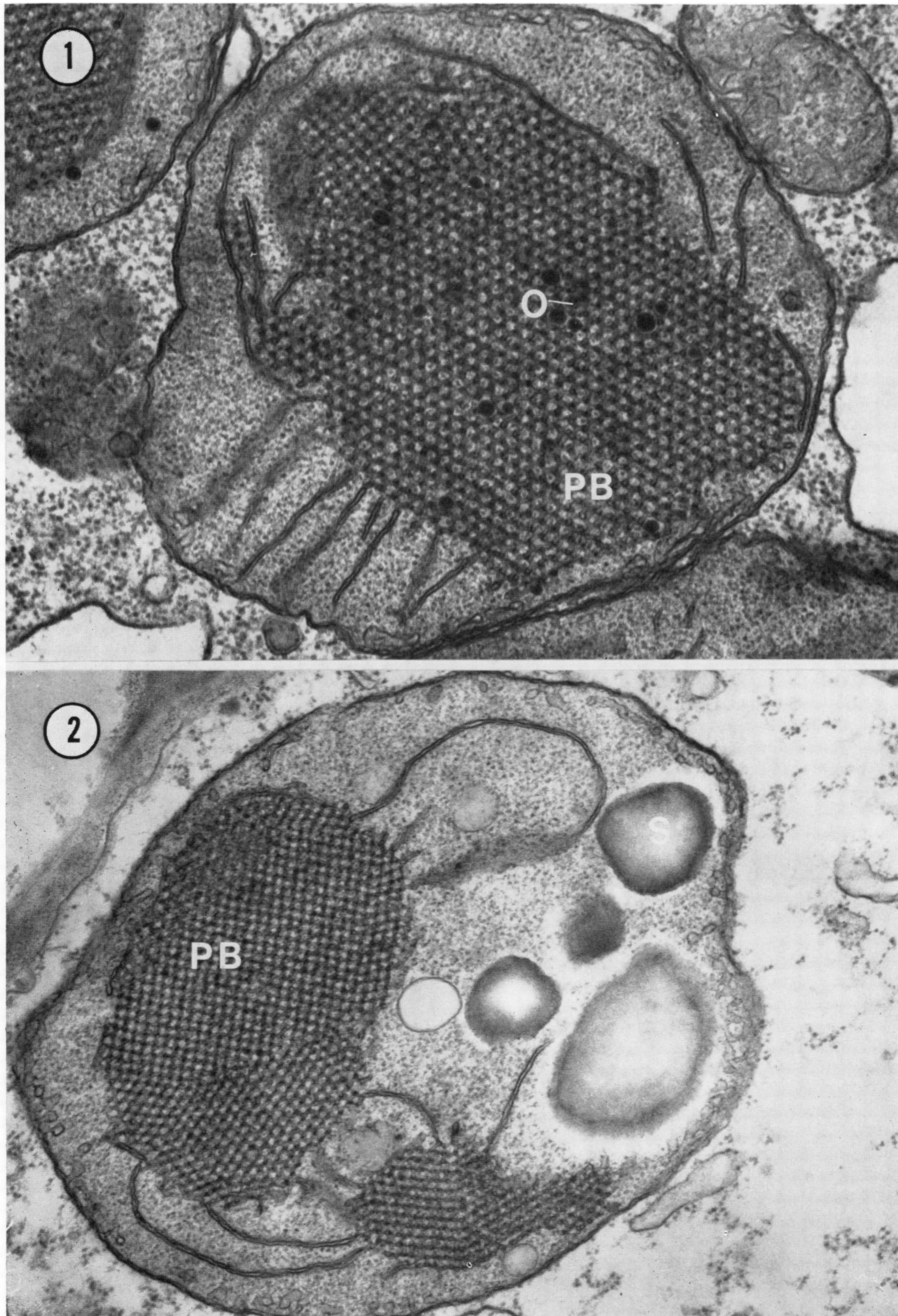

FIG. 1. Proplastid in leaf on wild-type (+/al) seedling grown for 1 week in darkness. Numerous osmiophilic bodies (○) are present within the prolamellar body (PB) and stroma. × 28,000.

FIG. 2. Proplastid in leaf on a seedling of the albescent mutant (al/al) grown in darkness for 1 week. Osmiophilic bodies are absent. Starch bodies (S) are present in the stroma. × 30,000.

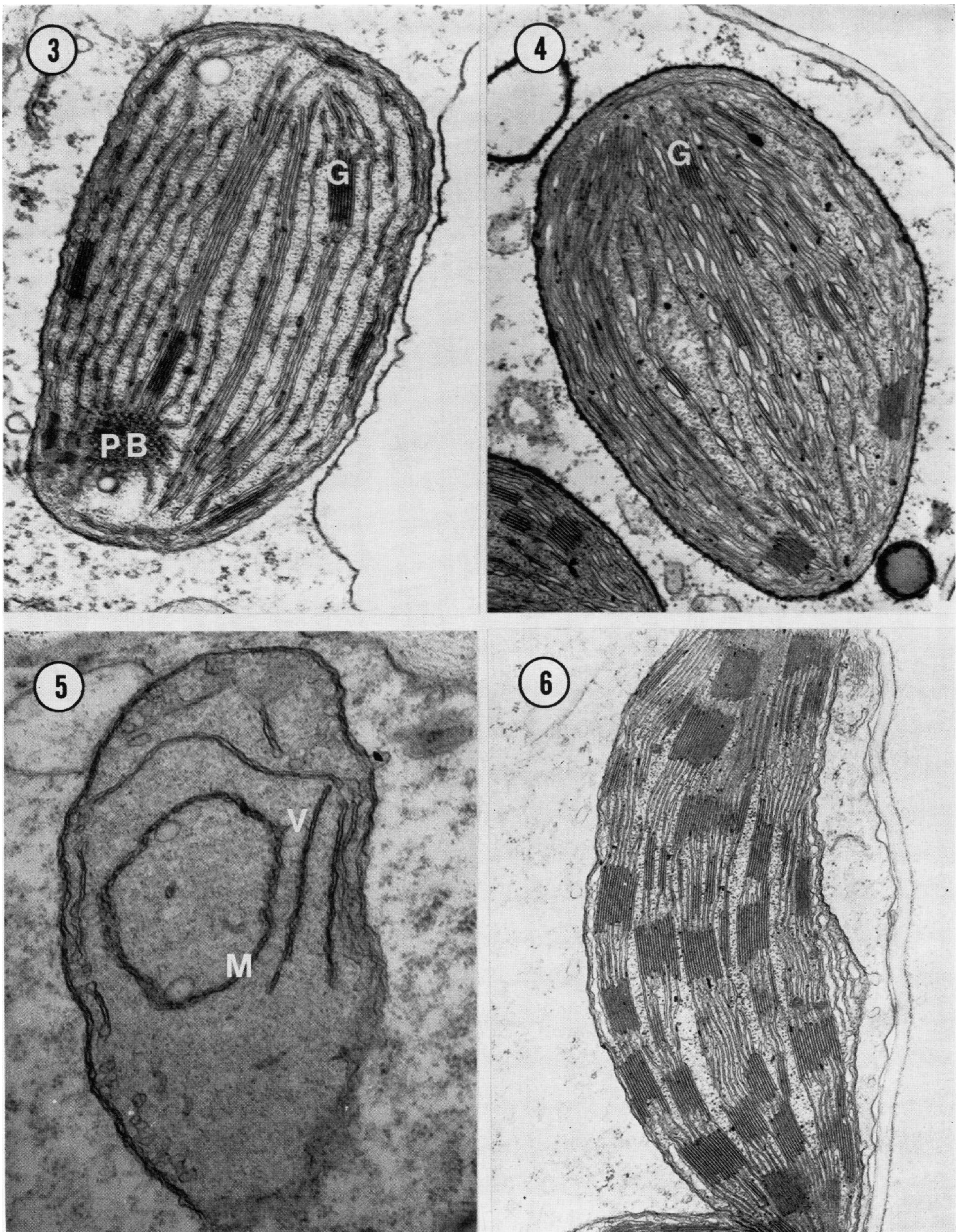

FIG. 3. Immature plastid in leaf of the albescent mutant grown for 1 week in low intensity light. A small portion of the prolamellar body (PB) remains and grana (G) formation is in progress. ×30,000.

FIG. 4. Developing plastid in wild-type seedling grown for 1 week in low intensity light. × 20,000.

FIG. 5. Section through leaf of 1-week-old albescent mutant grown under high intensity light. Swollen vesicles (V) and membranes (M) are located in the stroma. × 33,000.

FIG. 6. Plastid in leaf of albescent mutant grown for 1 week in low intensity light and illuminated for 30 hr in high intensity light. The fine structure of plastids in the mutant shown here was comparable to that in wild-type seedlings treated similarly. × 22,600.

trol leaves incubated on sucrose solutions did not produce additional protochlorophyllide-*a*. As anticipated (5, 10), little, if any, protochlorophyllide-*a* derived from ALA was photoreduced to chlorophyllide-*a* when ALA-fed leaves were placed in high intensity light. Prolonged illumination resulted in destruction of virtually all the ALA derived pigment.

Leaves detached from etiolated albescent and wild-type seedlings were placed on 0.2 M sucrose solutions lacking ALA and illuminated for 12 hr under high intensity light to determine the effect of ALA deprivation on pigmentation in excised leaves in light. Wild-type leaves synthesized chlorophyll-*a*, chlorophyll-*b*, and carotenoid pigment after a 4-hr lag period under these conditions (table II). Albescent leaves treated similarly turned white and spectrophotometric analyses of 80 % acetone extracts showed that they were pigmentless (table II).

Plants Grown in Low Intensity Light. Albescent and wild-type leaves on plants grown for 7 days under low intensity light were pale green by visual inspection. Partially differentiated plastids were observed in leaves on albescent (Fig. 3) and wild-type (Fig. 4) seedlings. The developing plastids in both genetic strains appeared to be quite similar in the electron microscope. In about 50 % of the sections examined, a portion of the prolamellar body was present in the developing plastids of either strain. Vesicle dispersal had occurred and grana formation was in progress at many sites in the stroma.

Chlorophyll-*a*, chlorophyll-*b*, carotene and xanthophyll concentrations were comparable in wild-type and mutant seedlings grown under low intensity light (table III). The pale green seedlings contained about one-sixtieth as much chlorophyll as was present in the wild-type grown from germination under high intensity light. Although xanthophyll concentrations in the wild-type were nearly twice that in etiolated seedlings, the level of carotenes changed very little (compare tables I and III). However, albescent seedlings grown in low intensity light contained as much carotene and xanthophyll pigment as did the wild-type. Since carotenoids were drastically reduced in the dark-grown mutant, a 15-fold increase in carotenes and a 28-fold increase in xanthophylls had occurred. Small quantities of colorless carotenoid precursors were still detectable in albescent seedlings grown under these conditions.

Plants Grown in High Intensity Light. Leaves on albescent seedlings grown for 7 days in high intensity light were white, whereas wild-type leaves were green and contained a normal plastid pigment complement (table IV). There was nothing unusual about the plastids in wild-type seedlings as revealed by electron microscopy. In the mutant, however, sac-like structures containing swollen vesicles and membranes were observed (Fig. 5) and recognizable plastids were lacking. From their size (*ca.* 2 × 5 μ), and their location in the peripheral cytoplasm of mesophyll cells, it was concluded that the sac-like structures were photo-bleached plastids.

To determine the functional competence, if any, of photo-bleached chloroplasts, detached leaves from bleached seedlings were incubated in darkness on solutions containing sucrose and ALA. After 12-hr treatments with ALA, the bleached leaves were reddish-brown upon visual inspection and displayed an intense red fluorescence under ultraviolet light (3600 A). The red pigments were extracted from

Table III. *Pigmentation in Albescent and Wild-type Leaves in Low Intensity Light*

Pigment concentrations are expressed on a gram-fresh weight basis. Representative data from 1 of 3 experiments is shown.

	Chlorophyll-*a*	Chlorophyll-*b*	Carotene	Xanthophyll
	mg/g × 10^{-3}	*mg/g* × 10^{-3}	*OD 470/g*	*OD 465/g*
1. (+/al)	2.8	0.8	3.1	8.2
2. (al/al)	2.6	0.8	3.0	8.4

Table IV. *Pigmentation in Variously Illuminated Albescent and Wild-type Leaves*

Values are expressed on a gram-fresh weight basis. Representative data from 1 of 3 experiments is shown.

Light conditions	Chlorophyll-*a*	Chlorophyll-*b*	Carotene	Xanthophyll
	mg/g × 10^{-2}	*mg/g* × 10^{-2}	*OD 470/g*	*OD 465/g*
High intensity				
1) (+/al)	17.0	5.8	14.0	28.0
2) (al/al)	0	0	0	0
Low-high intensity				
1) (+/al)	17.0	7.0	12.0	27.0
2) (al/al)	15.0	6.0	14.0	21.0

ground, ALA-treated leaves with ethyl acetate and glacial acetic acid (2:1). The extract was washed with 3 % sodium acetate 3 times and with water twice, and the red compounds were transferred from ethyl acetate to 15 % HCl. The spectrum of the HCl extract revealed an intense soret band at about 405 nm, and less intense peaks characteristic or porphyrins were observed between 548 and 598 nm. Further characterization of the porphyrins was not attempted. However, protochlorophyllide-*a* was not detectable in 80 % acetone extracts of ALA-fed leaves, suggesting that the porphyrins produced may not have been plastidic in origin. Alternatively, accumulation of porphyrins in photo-bleached leaves after ALA treatment could result from preferential destruction of plastid enzymes located in the lamellar membrane which catalyze the reactions between protoporphyrin IX and chlorophyll-*a*. This does not exclude the possibility that chlorophyll-*a* precursors from ALA to protoporphyrin IX normally arise by extraplastidic synthesis.

Plants Grown in Low and High Intensity Light. Pale green albescent seedlings grown in low intensity light produced normal chloroplasts in existing leaf tissue upon transfer to high intensity light. "Greening" was usually complete after about 24 hr under high light intensity conditions. Leaf tissue on albescent seedlings produced from the intercalary meristem after transfer to high intensity light turned white due to photo-bleaching of the chloroplast pigments. Transverse bands of green and white leaf tissue were produced on leaves of albescent seedlings illuminated under alternating 48-hr regimes of low and high intensity light, respectively.

The existing leaf tissue on albescent and wild-type seedlings produced comparable quantities of chlorophyll-*a*, chlorophyll-*b*, and carotenoids after transfer to high intensity light (table IV). The fine structure of chloroplasts in both strains illuminated in this fashion was normal. Fig. 6 shows a chloroplast in the mesophyll cell from an albescent seedling grown for 7 days in low intensity light and illuminated at high intensity for 30 hr. Evidently, wild-type and mutant seedlings were not adversely affected by prolonged exposure to low intensity light.

Discussion

The albescent mutant of maize is an interesting carotenoid-deficient variant. Etiolated seedlings are severely deficient in colored carotenoids and are photo-bleached when placed in high intensity light (table I, IV). This type of light sensitivity is characteristic of albino maize and sunflower mutants which are unable to synthesize the carotenoid pigments necessary to protect the photosynthetic apparatus (1, 6, 11, 19, 20). Although albino mutants described previously produce some chlorophyll when illuminated under low light intensities, plastid pigments are photo-bleached in these seedlings when exposed to high intensity light (1, 12, 19). In contrast to the light sensitive albino mutants, albescent maize pretreated with low intensity light produced the normal plastid pigment complement during subsequent exposure to high intensity light (table IV) (16).

Proplastids in wild-type and mutant seedlings as revealed in the electron microscope are quite similar (Fig. 1, 2). The size of prolamellar bodies and the concentration of photo-convertable protochlorophyllide-*a* were comparable in both genetic strains (table I). This is consistent with previous descriptions of the prolamellar bodies and protochlorophyllide-*a* levels in proplastids of albino sunflower and maize mutants (3, 20). Evidently, deranged carotenoid metabolism has little, if any, effect on the metabolic pathway for chlorophyll biosynthesis. This was explored further in experiments on ALA utilization in detached, dark-grown leaves from albescent maize seedlings. Synthesis of protochlorophyllide-*a* from ALA occurred in comparable amounts and at comparable rates in wild-type and mutant leaves (table II). From these data it was concluded that mutation at the albescent locus does not affect the metabolic pathway for chlorophyll synthesis adversely.

One significant structural difference was found between albescent and wild-type proplastids. Osmiophilic bodies present in the wild-type were lacking in all sections of albescent proplastids examined. This correlates nicely with von Wettstein's suggestion that these structures may serve as storage centers for carotenoid pigment in proplastids (21). Since osmiophilic bodies are round, and do not appear to be associated with a limiting membrane (Fig. 1), they probably consist of substances in a liquid phase system (*e.g.* oil droplets). Small has shown that non-polar lipids such as β-carotene (mp 180°) form non-spreading solids in water at room temperature (18). Therefore, if carotenoids are present in osmiophilic bodies, other compounds with lower melting points (*e.g.* triglycerides) must also be present to render the carotenoids soluble at room temperature. This suggests that proplastids in albescent maize may be deficient in substances other than carotenoid pigments. Nevertheless, the concomitant growth of osmiophilic bodies during δ-tocopherylquinone accumulation in senescent tobacco and maple leaves (4), and during chlorophyll accumulation in a sunflower mutant unable to make grana (20) exemplify probable relationships between lipid composition and plastid ultrastructure. Conversely, it would not be surprising that a deficiency in carotenoids in albescent maize might result in recognizable loss of structures considered to be storage centers for these compounds (21).

Seedlings of albescent maize grown under low intensity light contain chlorophyll-*a*, chlorophyll-*b* and carotenoids in amounts equivalent to that in the wild-type treated similarly (table III). Since the dark-grown mutant is severely deficient in colored

carotenoids, considerable synthesis of these compounds had occurred in seedlings grown in low intensity light. Thus, dark-inhibited carotenoid synthesis was initiated during this treatment. This suggests the possibility that a photo-labile inhibitor of carotenoid synthesis may exist in the dark-grown mutant. A more compelling alternative might be that there are normally 2 metabolic pathways for carotenoid synthesis in higher plants, *i.e.,* one pathway operating in darkness and the other in light. Persistence of carotenoid precursors (tentatively identified as phytoene and phytofluene) in the mutant acetively synthesizing colored carotenoids under low intensity illumination would support the latter possibility.

The effects of light on carotenogenesis in albescent maize deserves further comment. Leaves on the wild-type and mutant produce comparable quantities of carotenoid pigment following a suitable light regime. However, in the mutant virtually all of the carotenoid pigment is produced in light, whereas in the wild-type strain, about 20 % of the carotenoid pigment is made in darkness and 80 % is made in light. This suggests that if there are separate pathways for carotenoid synthesis in darkness and in light, the pathway in light can compensate for the initial deficiency in the mutant, since the final carotenoid pigment concentrations in both strains are ultimately equivalent.

The action spectrum for carotenoid synthesis in a *Chlorella* mutant displayed peaks at 675 and 420 nm (7). In general, this action spectrum resembles the absorption spectrum of chlorophyll-*a* with reduced effectiveness in the 420 nm region presumably due to carotenoid screening. The action spectrum for carotenogenesis in higher plants has been difficult to determine due to the high concentration of carotenoid normally present in etiolated seedlings, and to the lack of mutants suitable for this purpose. It can be anticipated that these difficulties will be overcome through the employment of albescent maize, because the concentration of carotenoid pigment in the etiolated mutant is low, normal levels of plastid pigment are finally attained in suitably pre-illuminated seedlings, and light is required for virtually all of the carotenoid pigment produced.

Finally, existing leaf tissue on albescent seedlings grown in low intensity light produced normal chloroplasts and accumulated normal quantities of plastid pigments when placed in high intensity light. New leaf tissue derived from the intercalary meristem was white due to photo-bleaching of any pigment which may have been produced. These results may be explained by the failure of the leaf tissue produced after the seedlings were placed in high intensity light to synthesize carotenoid pigment in amounts and at rates necessary to protect the chlorophyll synthesized under these conditions. It is of interest that in wild-type seedlings grown at low light intensity there was little net synthesis of carotenoid pigment and that the similarity illuminated mutant produced about the same quantity of carotenoid as was present in the wild-type. This suggests that carotenoid synthesis in both strains in light is intensity dependent, and that the carotenoid concentration in the etiolated wild-type necessary for protecting plastid pigments from photo-bleaching may be about the same as that in the mutant.

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