Etioplast Development in Dark-grown Leaves of *Zea mays* L.[1]

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ABSTRACT

The ultrastructure of etioplasts and the acyl lipid and the fatty acid composition of sequential 2-centimeter sections cut from the base (youngest) to the top (oldest) of nonilluminated 5-day-old etiolated leaves of *Zea mays* L., and the acyl lipid and fatty acid composition of the etioplasts isolated from them have been investigated. There is a 2.5-fold increase in the size of the plastids from the base to the tip of the leaf, and an increase both in the size of the prolamellar body and in the length of lamellae attached to it. The etioplasts in the bundle sheath and mesophyll cells of the older, but not the younger leaf tissue, are morphologically distinct. The monogalactosyl and digalactosyldiglycerides, phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol were the only detectable acyl lipids in the isolated etioplast fractions. Together with phosphatidylethanolamine these were also the major acyl lipids in the whole leaf sections. With increasing age of the leaf tissue, increases occurred in two of the major plastid lipids, monogalactosyldiglyceride and phosphatidylglycerol, while the levels of essentially nonplastid lipids remained constant or declined slightly. The monogalactosyldiglyceride to digalactosyldiglyceride ratio increased from 0.4 to 1.1 in the tissue sections of increasing age and from 0.7 to 1.2 in the etioplasts isolated from them. Similarly, the galactolipid to phospholipid ratio increased from 0.8 to 1.4 in the tissue and from 0.5 to 4.5 in the isolated plastids. In the latter, the proportions of phosphatidylglycerol (as a per cent of total phospholipid) increased from 20 to 41% with increasing age of plastids.

Linolenic acid was the major fatty acid in the total lipid of each of the etioplast fractions, but it was only the major fatty acid in the total lipid of the oldest leaf tissue. Its proportion in both total lipid extracts and individual lipids increased with age. The *trans* Δ³ hexadecenoic acid was absent from all lipids. The protochlorophyllide content of the tissue increased with age. The results are discussed in relation to the use of illuminated etiolated leaves for studying chloroplast development.

Although greening etiolated leaves, illuminated isolated etioplasts, and isolated etiochloroplasts (plastids from a greening etiolated leaf) are used extensively to study chloroplast development, relatively little is known about the cells of etiolated leaves or, more importantly, of the etioplasts within them.

The assumption, until recently, seems to have been that the etioplast population within a leaf is homogeneous. Two observations (prior to 1971) suggest that this may not be so: Laetsch and Price (13) observed that in etiolated sugarcane leaves, the etioplasts in the bundle sheath and mesophyll cells were morphologically distinct, and Leech *et al.* (15) found differences in the fatty acid composition of sequential 4-cm segments of 7-day-old maize seedling shoots. The observation of Laetsch and Price (13) is

significant only if the developmental sequence for bundle sheath and mesophyll etioplasts in sugarcane follows a similar sequence to that of bundle sheath and mesophyll chloroplasts in light-grown maize: that is, that the dimorphic chloroplast population differentiates from a monomorphic one, which is found in the youngest leaf tissue (14). The implications of the finding that the fatty acid composition of etiolated tissue changes with age are particularly important to those investigations examining changes in composition following illumination.

Subsequently, Robertson and Laetsch (20) have shown that there is both a morphological and a biochemical development of etioplasts from the base to the tip of an etiolated barley leaf; the "oldest etioplasts" developed into fully functional chloroplasts more rapidly than did younger etioplasts when the leaves were illuminated. Changes in the composition of whole etiolated barley leaves of different ages have also been reported (25).

There is still no precise information on the homogeneity or otherwise of the lipid composition of either a single etiolated leaf or of the etioplasts within them. This is of considerable importance to those studies in which the composition of whole etiolated leaves or of the etioplasts/etiochloroplasts isolated from them is monitored during greening and to the conclusions that are drawn, particularly those made in relation to chloroplast and thylakoid development.

The monocotyledon leaf, because of its mode of growth, has shown itself to be an excellent system for the study of plastid development in both green (14, 16) and etiolated leaves (20). This paper reports an investigation on the ultrastructure and the lipid and fatty acid composition of sequential segments of etiolated maize leaves and of the lipid and fatty acid compositions of the etioplasts isolated from them.

MATERIALS AND METHODS

The System. Five-day-old seedlings of *Zea mays* L. var. Kelvedon Glory which had been grown in total darkness at 27 C were used in these experiments (17). At this age there were three obvious leaves, which were either still contained within the coleoptile, or were just emerging from it. The oldest (first) leaf was about 6 to 8 cm in length and did not have a sheath. No attempt was made to separate the leaves since it was assumed that as the leaves grow from a basal meristem, the plastids at a given distance from the leaf base in each of the three leaves would be at the same stage of development (*cf.* ref. 15).

The leaves (plus coleoptile) were excised at the coleoptilar node (Fig. 1). The coleoptile was removed for all experiments except for plastid isolation.

All measurements of distance along the leaf were made from the base of the leaf (youngest tissue) toward the leaf tip (progressively older tissue).

For electron microscopy transverse sections 1 mm wide were taken at 1-cm intervals along the leaf. For all other experiments the leaves were cut into three sequential 2-cm sections which were termed sections X (proximal section), Y, and Z, respectively, and represented tissue of increasing age. The plastids isolated from
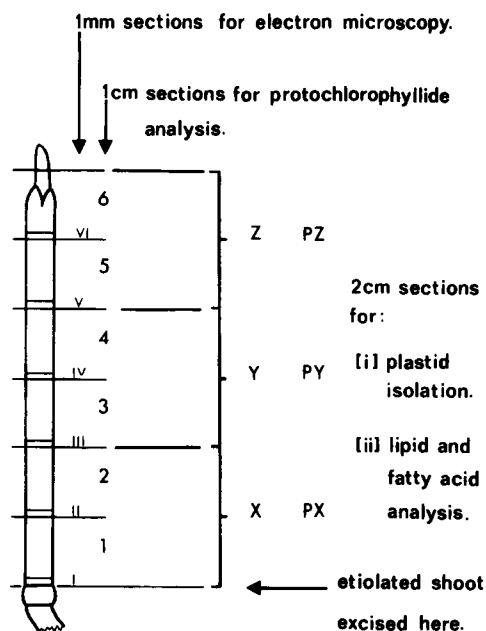
FIG. 1. Diagram of 5-day-old dark-grown seedling of *Z. mays* L. showing how the plant shoot was sectioned and the purpose for which each section was used. Diagram shows tip of oldest leaf just emerging from coleoptile.

these three sections were called fractions PX, PY, and PZ, respectively.

The leaf was divided into six 1-cm sections for Pchl(ide) determinations.

Plastid Isolation. Plastids were isolated and washed by the method of Leese *et al.* (17) except that initially the tissue was blended for two periods of 10 sec.

Electron Microscopy. Leaf tissue was cut directly into 2.5% (v/v) glutaraldehyde in Na_2HPO_4/KH_2PO_4 buffer (pH 8.0) containing 1 mM $MgCl_2$ and 0.2% (w/v) BSA (Coln fraction V; Koch light). The 1-mm-wide sections were cut into 2-mm lengths and transferred to stoppered vials containing the same buffered fixative solution, and left for 1 hr at room temperature. The sections were postfixed for 1 hr at room temperature in 1% (w/v) osmium tetroxide in the same buffer, dehydrated in a graded acetone series, and embedded in Spurr's resin as described elsewhere (15). All fixation, dehydration, and embedding manipulations were carried out as quickly as possible in dim green light (25-w tungsten lamp in an Ilford safelight fitted with a 905 X filter); otherwise the samples were kept in complete darkness.

Lipid Extraction and Analysis. Lipids were extracted from whole tissue by cutting it into small pieces directly into chloroform-methanol (1:1, v/v) and heating briefly to 60 C (7). Tissue was left to extract overnight (8–12 hr) at room temperature; the solvent was decanted and the tissue reextracted three times (30 min each time with an initial brief heating to 60 C), twice with chloroform-methanol (2:1, v/v) and once with pure chloroform. The four extracts were combined and washed for 3 hr at 0 to 4 C with 20% of their total volume of 100 mM NaCl. The lower solvent phase was removed and evaporated to dryness under vacuum at 30 C. The dried extracts were taken up in chloroform and re-evaporated twice more. Extracts were stored dry under N_2 at −20 C in darkness. The initial stages of tissue extraction (*i.e.* cutting and heating) were carried out in dim green light. Subsequent manipulations were performed in the light. At all other times, the tissue being extracted and the decanted extracts were kept in total darkness.

The lipids of suspensions of plastids were extracted by methods described previously (19) for the extraction of lipids from membrane fractions.

Individual lipids were separated from total lipid extracts by TLC using two different one-dimensional solvent systems: either chloroform-methanol-acetic acid-water (85:15:10:4, v/v/v/v) or toluene-ethylacetate-ethanol (2:1:1, v/v/v). Lipids for quantitation were located with iodine vapor, which was allowed to evaporate from the thin layer plates before analysis. Galactolipids and phospholipids were quantified as described previously (19) by the methods of Roughan and Batt (21) and Bartlett (2), respectively.

Total phospholipid in the total lipid of whole tissue was estimated as the phosphate remaining at the origin after chromatography of the extract in toluene-ethyl acetate-ethanol (2:1:1, v/v/v). Since only three phospholipid spots were evident on the chromatograms of the plastid lipid extracts, total phospholipid was taken as the sum of the individual phospholipids present.

Fatty Acid Preparation and Analysis. Lipids for fatty acid analysis were separated by two-dimensional TLC using the solvent system of Gardner (6) and were located by spraying with 0.2% (w/v) 2',7',-dichlorofluorescein in 50% (v/v) ethanol, and visualizing under UV light.

Fatty acid methyl esters of the total lipid extract and of individual lipids were prepared and analyzed as described previously (19).

Pchl(ide) Extraction. Pchl(ide) was extracted from fresh tissue by grinding with a little acid-washed sand in 80% acetone, and quantified spectrophotometrically using the equations derived by Horton (8). All manipulations were carried out in dim green light as already described.

RESULTS

Electron Microscopy. The ultrastructure of etioplasts *in vivo* in a developing dark-grown maize leaf was investigated by examining ultra-thin sections cut from 1-mm transverse sections (I–VI) excised from the whole leaf at 1-cm intervals (Fig. 1). The sections were cut in the transverse plane of the leaf so that it was easy to distinguish between mesophyll and bundle sheath cells. Electron micrographs, representative of the different stages in the ultrastructural development of the etioplasts, are shown in Figure 2. Measurements made from electron micrographs are given in Table I.

The plastid profiles in the two cell types were not readily distinguishable in the lower sections, *i.e.* 0 to 2 cm from the leaf base (Fig. 2, A through D). In this region of the leaf the mean length of the plastid profiles increased from 1.9 (I) to 2.8 μm (II and III) while the mean width, increased from 1.5 (I) to 1.8 μm (III) (Table I), giving a L:W ratio of about 1.4.

The plastid profiles at the leaf base (section I) were of two types: either they had a very granular stroma, an occasional discrete lamellae membrane, and a few plastoglobuli (Fig. 2A), or they had a very electron-dense stroma a few starch grains and discrete lamellae (Fig. 2B). Only rarely did either plastid type contain a prolamellar body. The plastids in section II resembled amyloplasts (Fig. 2C); they had a more granular stroma, more starch grains, only an occasional PLB,[2] and a few discrete lamellae, which were often in close association with the starch grains.

The plastid profiles in section III (2 cm from leaf base) (Fig. 2D) were recognizable as etioplasts, most profiles contained a PLB, the number of starch grains was very much reduced, and the lamellae membranes which were present sometimes had a beaded appearance and, whereas in younger plastids these membranes were discrete, here they appeared to be attached to the prolamellar bodies.

The differences between the etioplasts in the bundle sheath and

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[2] Abbreviations: MGDG: monogalactosyldiglyceride; DGDG: digalactosyldiglyceride; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; GL: total galactolipid; PL: total phospholipid; PLB: prolamellar body; TFA: total fatty acids.
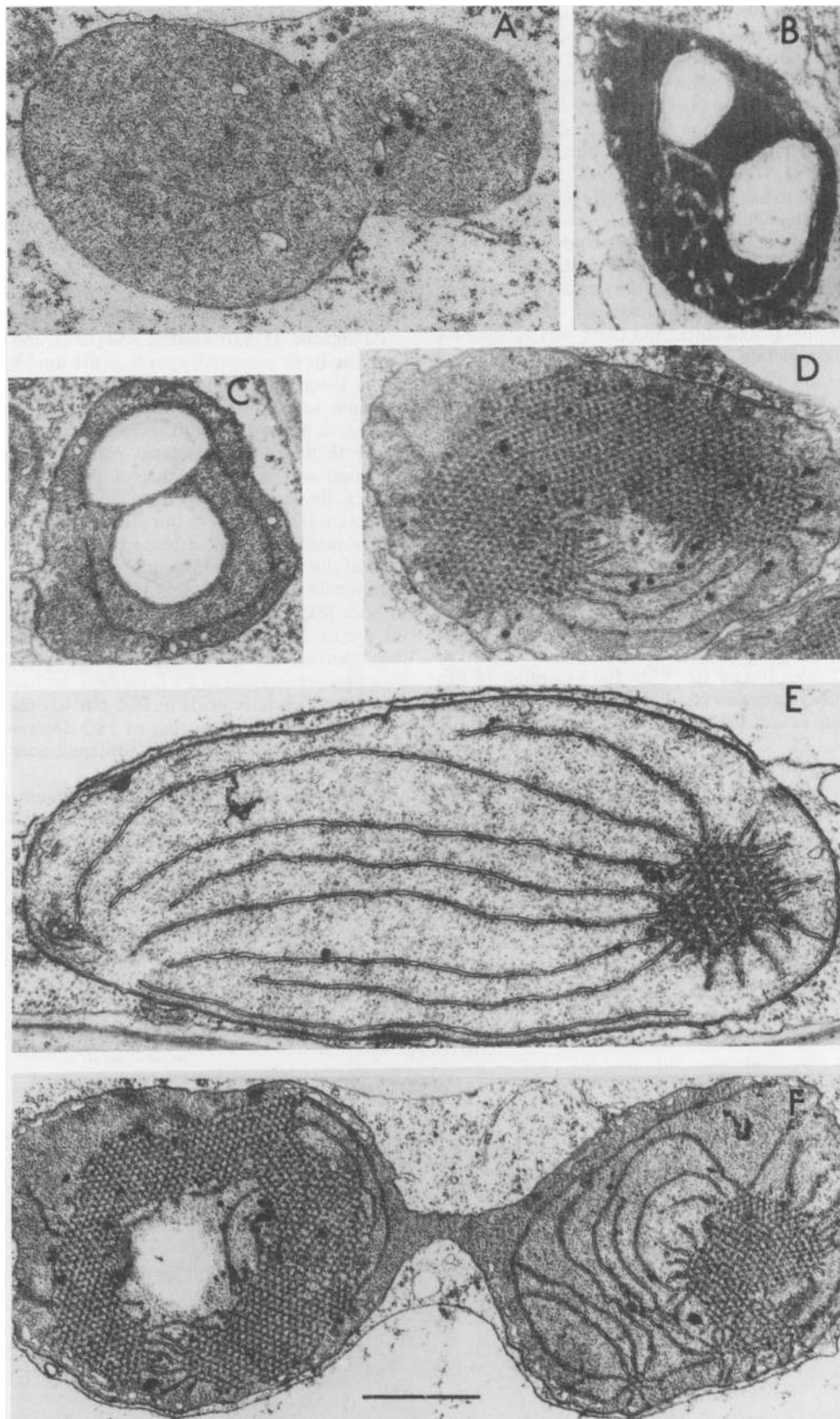
FIG. 2. Representative electron micrographs of plastids in cells along an etiolated maize leaf. A and B: plastids from cell at leaf base; C: plastids in cell 1 cm from leaf base; D: plastid in cell 2 cm from leaf base; E: plastid from bundle sheath cell 3 cm from leaf base; F: dumbbell-shaped plastid profile from mesophyll cell 2 cm from leaf base. A ($\times$ 20,300); B ($\times$ 23,600); C ($\times$ 22,300); D ($\times$ 23,000); E ($\times$ 24,100); F ($\times$ 18,000).

mesophyll cells were more obvious in the older leaf tissue 3 to 5 cm from the leaf base (sections IV–VI). Both the mean profile length and width of the mesophyll plastids increased by 60% between sections III and VI, but while a similar increase occurred in the length of the bundle sheath plastid profiles their width only increased by 18%. Thus, those in the mesophyll cells had a more rounded profile (L:W = 1.4) while those in the bundle sheath cells were more elongated (L:W = 2.0). Moreover, the mesophyll etioplast profiles tended to have a more centrally placed PLB with attached discrete lamellae which very often formed loops which joined two distinct regions of the same PLB. In contrast the bundle sheath etioplast profiles (Fig. 2E) had polar prolamellar bodies and several discrete lamellae transversing the length of the profile.

These older plastids had only the occasional starch grain, a more developed peripheral reticulum, and there was an increase in the number of plastoglobuli.

Two types of apparent plastid division were observed. In several profiles of young plastids (section I), a slight constriction was seen along the plastid length at which point a double membrane was present at right angles to the long axis of the plastid (Fig. 2A). In older tissues, characteristic dumbbell-shaped plastid profiles were observed (Fig. 2F).

The envelope membranes were similar to those delineating other plastids. Only very rarely, even in the profiles of the youngest plastids (I), did the inner envelope membrane appear to be continuous with the internal membranes of the plastid.

Acyl Lipid and Fatty Acid Analysis of Etiolated Leaf Sections and Isolated Etioplasts. The acyl lipid composition of etiolated leaf sections of increasing age and of the plastids isolated from them is shown in Tables II and III. With the exception of PE, which was found only in extracts of whole leaves, the major acyl lipids of both the leaves and the isolated plastids were: MGDG, DGDG, PC, PG, and PI.

With increasing age the ratio between the MGDG and the DGDG nearly tripled from 0.4 to 1.1 in whole tissue, and nearly doubled from 0.7 to 1.2 in the corresponding plastid fractions. The increase in the ratio between GL (MGDG + DGDG) and PL was much smaller in whole leaf tissue of increasing age (0.8–1.3) than in the isolated plastids (0.5–4.5) (Table II).

PC was the major single phospholipid in all of the tissue sections, although its relative proportion (as per cent of PL) varied from section to section (Table II). PG was also present in all of the leaf sections but its proportion of PL increased with the age of the tissue. PC and PG were the two major phospholipids in the isolated plastid fractions and together they constituted more than 80% of the PL in each of these fractions. However, the proportion of PC declined from 64% (PX) (youngest etioplasts) to 40% (PZ) while that of PG increased from 20% (PX) to 41% (PZ).

Calculations of the lipid content of sequential 2-cm sections of the *first* leaf showed that MGDG and PG (in nmol/section) increased from the base to the tip of the leaf, while DGDG, PC, and PE all decreased initially and then increased again to their initial levels, towards the leaf tip (Table III).

Palmitic acid (16:0), linoleic acid (18:2), and linolenic acid (18:3) were the three major fatty acids in the total lipid extracts of both whole tissue and the isolated plastids (Table IV). Linoleic and linolenic acids were the two fatty acids showing the greatest change in proportion with increasing age of the tissue. In both whole tissue and the isolated plastids the proportion of 18:3 increased with age while that of the 18:2 decreased. However, whereas linolenic acid was the major fatty acid of the three plastid extracts, it was only the major fatty acid in the extract of the oldest leaf section, linoleic acid being the major fatty acid in the total lipid of the younger leaf sections (X and Y).

In whole tissue of all ages 18:3 was the major single fatty acid in both MGDG and DGDG, and its proportion increased with the age of the tissue. Linoleic was the major fatty acid in PC but its proportion declined slightly with age and this was accompanied by a parallel increase in 18:3. Palmitic acid and linoleic acid were the two major fatty acids in PG, but whereas the proportion of 16:0 increased with age, that of 18:2 decreased with age. There was no trace of the *trans* Δ^3 hexadecenoic acid in either PG or any of the other lipids (Table V).

Pchl(ide) Content. The Pchl(ide) content of sequential 1-cm sections of an etiolated leaf increased 3-fold from the base to the middle of the leaf and a further 2-fold from the middle to the tip of the leaf (Table VI). Only one species of Pchl(ide) with a broad peak at 635 nm was detectable in glycerol extracts of these sequential sections of etiolated maize leaves (66% v/v glycerol in etioplast isolation medium). Illumination of these extracts for 20 sec with a 150-w tungsten filament lamp (4.4 × 10^4 ergs sec^{-1} cm^{-2}) filtered through 17 cm of distilled H_2O resulted in the

Table I. The mean dimensions of etioplasts of different ages in leaves of dark grown *Zea mays* L.

Leaf section	Distance of sample from leaf base	LEAF CELL TYPE					
		MESOPHYLL			BUNDLE SHEATH		
		Length (L)	Width**(W)	L/W	Length (L)	Width (W)	L/W
	(cms)						
I	0.0 – 0.1	1.9	1.5	1.3	–	–	–
II	1.0 – 1.1	2.8	1.8	1.5	–	–	–
III	2.0 – 2.1	2.8	2.0	1.4	–	–	–
IV	3.0 – 3.1	3.4	2.5	1.4	4.0	1.8	2.2
V	4.0 – 4.1	4.2	3.2	1.3	4.7	2.4	2.0
VI	5.0 – 5.1	4.5	3.2	1.4	4.4	2.3	1.9

* see Fig. 1 for details ** width measured at L/2 – no ready distinction between bundle sheath and mesophyll plastids

Measurements were made on up to 50 mesophyll plastid profiles and up to 20 bundle sheath profiles to obtain the means recorded here.

Table II. The galactolipid and phospholipid composition (mole:mole or mole%) of etiolated leaf sections and of the plastids isolated from them

Plastids or Tissue	Leaf section cms from base	MGDG:DGDG[2]	GL:PL	Phospholipid composition		
				PC	PG	PI	PE
		molar ratios		moles %		
Plastids (3)*	X (0 – 2)	0.7	0.5	64	20	16	0
	Y (2 – 4)	1.1	2.8	50	32	17	0
	Z (4 – 6)	1.2	4.5	40	41	18	0
Tissue (4)*	X (0 – 2)	0.4	0.8	48	7	–	19
	Y (2 – 4)	0.9	0.8	52	10	–	27
	Z (4 – 6)	1.1	1.4	42	11	–	17

* The data given are the means for the number of analyses shown in parentheses

0 absent; – present but not determined

PL = PC + PG + PI for isolated plastids = phosphate remaining at origin after one dimensional chromatography in toluene : ethyl acetate : ethanol (2:1:1 v/v/v) for tissue samples

Table III. The galactolipid and phospholipid composition of etiolated leaf sections (nmoles/leaf section)

Leaf section (cms from base)	MGDG	DGDG	PC	PG	PE
	nmoles/2 cm section of first leaf				
X (0 – 2)	19.5	48.7	40.9	6.0	16.2
Y (2 – 4)	25.1	27.9	34.4	6.6	13.9
Z (4 – 6)	64.0	58.2	39.5	10.3	16.0

Table IV. The fatty acid composition of the total lipid extracted from etiolated leaf sections and of the plastids isolated from them

Plastids or Tissue	Leaf section (cms from base)	16:0	16:1[9]	18:0	18:1	18:2	18:3
		moles %					
Plastids	X (0 – 2)	19.8	2.4	4.7	16.2	20.3	31.6
	Y (2 – 4)	17.6	3.6	4.8	11.1	15.9	44.5
	Z (4 – 6)	16.7	1.5	3.8	7.1	15.1	53.2
Tissue	X (0 – 2)	19.1	0.7	1.2	5.1	55.4	17.4
	Y (2 – 4)	19.4	0.7	1.4	4.6	40.2	32.0
	Z (4 – 6)	20.7	1.0	2.0	7.0	30.5	37.9

There were also small proportions of fatty acids with a chain length 16C, and 16:2 and 16:3 fatty acids present, which were included for the calculation of the molar compositions given above but which have not been entered in the table of results

Table V   The fatty acid composition of the MGDG, the DGDG, PC and PG extracted from sequential 2 cm etiolated leaf sections of Zea mays L.

Lipid	Leaf section (cms from base)	16:0	16:1 9	18:0	18:1	18:2	18:3
				moles %			
MGDG	X (0-2)	5.9	0.9	1.5	4.1	15.1	71.5
	Y (2-4)	7.4	1.1	2.0	3.9	6.6	76.3
	Z (4-6)	5.9	0.4	1.9	2.0	5.0	84.9
DGDG	X (0-2)	12.8	0.3	1.2	5.7	23.1	54.0
	Y (2-4)	13.0	1.0	2.0	8.3	13.7	55.8
	Z (4-6)	7.9	0.4	1.4	4.3	6.8	76.1
PC	X (0-2)	21.6	0.6	2.1	8.1	61.0	3.8
	Y (2-4)	20.2	0.6	1.9	6.0	59.8	9.1
	Z (4-6)	24.4	0.5	0.4	3.2	52.7	15.0
PG	X (0-2)	35.1	0.7	2.2	7.4	39.1	10.9
	Y (2-4)	40.5	1.0	1.7	10.1	30.9	9.9
	Z (4-6)	45.4	1.5	1.8	3.7	21.6	20.7

There were also small proportions of fatty acids with a chain length < 16C, and 16:2 and 16:3 fatty acids present which were included for the calculation of the molar compositions given above but which have not been entered in the table of results

Table VI   The protochlorophyllide content (µg/section) of sequential sections (1 and 2 cm) of etiolated maize leaves

	Leaf Section*					
	X		Y		Z	
	1	2	3	4	5	6
			µg			
Protochlorophyllide/1 cm sect.	0.06	0.13	0.20	0.32	0.40	0.50
Protochlorophyllide/2 cm sect.	0.19		0.52		0.90	
Protochlorophyllide/2 cm sect. /mg FW 1st leaf	0.10		0.29		0.56	
1st leaf	0.004		0.008		0.015	

* See Fig. 1 for explanation

Protochlorophyll(ide) in mmoles/l = $33.22 A_{626} - 4.50 A_{663} - 7.58 A_{645}$
(See reference 8 for derivation)

formation of two peaks: a small one at 625 and a much larger one at 672 nm.

DISCUSSION

The electron micrographs of plastids in cells along the length of an expanding etiolated leaf clearly show that as the tissue increases in age there is an increase in both the size and ultrastructural complexity of the plastids. Similarly, the analyses of the lipids, fatty acids, and Pchl(ide) of both intact tissue and the corresponding etioplast fractions indicate that a biochemical development of the plastids also occurs along the etiolated leaf. The results fully substantiate the findings of Robertson and Laetsch (20) who observed both a biochemical and an ultrastructural development in etioplasts along the length of an etiolated barley leaf, and they also confirm the observations of Laetsch and Price (13) and Leech et al. (15).

Changes in the ultrastructure of the mesophyll plastids along the length of these 5-day-old etiolated maize leaves are similar to those reported for etiolated barley by Robertson and Laetsch (20). These include the increase in the size of the PLB and its central position within the plastid profile, and the increase in the length of lamellae membranes attached to the PLB. A major difference is that the profiles of these maize proplastids appear to be about twice the diameter of those in barley.

There are also several interesting comparisons to be made between the observations reported here and those that have been made on etioplast development in the primary leaves of dark-grown Phaseolus vulgaris L. Despite discrepancies on the actual timing of the appearance of the PLB in this species (after 3 days, Weier and Brown [27]; after 9 days, Bradbeer et al. [4]), points of similarity between the development in the two species are: the formation of amyloplasts (4, 27–29), the subsequent disappearance of starch with the seemingly parallel increase in membrane, the rounded appearance of the plastid profiles, and the rapid increase in the size of both the plastid and the PLB at a precise stage in the

developmental sequence. Differences between the systems include a lack of association of ER with the etioplasts in maize (Whatley [29] also noted that this association was not as evident in maize as it was in bean) and the apparent absence of invaginations of the inner envelope membrane in maize. In bean it was thought that the internal lamellae were derived from these invaginations (4, 27). A further difference appears to be one of the time scale of the development, in maize the time difference between defined developmental stages is in hours whereas in bean it can be in days.

The increases in the over-all dimensions of the plastids with increasing age of the tissue in these 5-day-old etiolated leaves are also similar to those in the first 4 cm of a 7-day-old light-grown maize plant (14, 15); however, whereas the chloroplasts continue to increase in size beyond this stage, the etioplasts (sections V and VI) remain a constant size. Increases in the numbers of plastoglobuli with age in etioplasts without apparent membrane loss suggest continuing lipid synthesis in the absence of membrane synthesis.

Until relatively recently plastid division was thought to be light-dependent (3, 11). The results of Robertson and Laetsch (20) who were able to show an increase in plastids/cell along etiolated barley leaves (i.e. with age) and Bradbeer et al. (4) who were able to show an increase in the total number of plastids in primary leaves of etiolated P. vulgaris L. of increasing age, confound this view. Dumbbell-shaped plastid profiles and plastid profiles with a central baffle at right angles to the long axis of the plastid have both been interpreted as division profiles (5). Both types of profile were observed here, the latter was confined to the proplastids in the very youngest tissue sampled while the former was observed in several sections along the leaf, particularly those 2 and 3 cm from the leaf base. If the interpretations of these profile shapes are correct this implies two phases of division in dark-grown maize leaves, each with its own division mechanism. Two phases of plastid division were also noted in etiolated primary leaves of bean by Bradbeer et al. (4), one between days 4 and 7 and the other between days 9 and 12 of growth. The mechanism of the first division was not apparent while the second was thought to occur by plastid constriction.

The ultrastructurally distinct plastids occurring in the more mature mesophyll and bundle sheath cells are similar to those observed in mature leaves of etiolated sugarcane (13). That the bundle sheath etioplasts contain discrete lamellae arranged in exactly the same way as in fully differentiated bundle sheath chloroplasts raises the question as to whether chloroplast development in these cells is the same during greening as it is in normal light-grown leaves. The granal chloroplast stage which precedes the dedifferentiation step (14, 15) in the normal light system would appear to correlate with the single morphological type of etioplast which precedes the differentiation into the two etioplast types. These two possible pathways of development are summarized in Figure 3.

The acyl lipid and fatty acid composition of etioplasts and etiolated tissue are, with the exception of the trans Δ^3 hexadecenoic acid, which is absent from these tissues and plastids, qualitatively similar to those of isolated chloroplasts of green tissue (10, 14, 16, 23).

The increases in the levels of MGDG and PG (found predominantly but not exclusively in plastid membranes [19]) in tissue of increasing maturity correlates well with the increases in the over-all size of the plastids and particularly with the size of the PLB. The levels of lipids characteristic of extrachloroplastic membranes (e.g. PC and PE), on the other hand, either remain constant or show a slight decrease along the lamina of the etiolated leaf. The analyses of the isolated plastids confirm that the major lipid changes occurring in the leaf are located in the etioplasts. The absence of PE in the plastid fractions is an indication of the absence of contaminating extrachloroplastic membranes, which contain relatively high proportions of it.
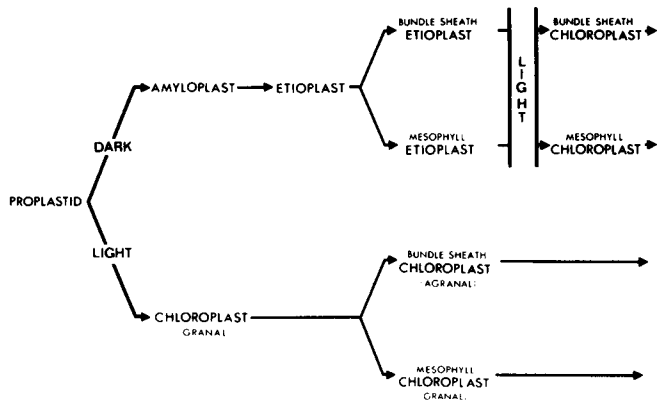
FIG. 3. Diagram comparing sequence of plastid development in bundle sheath and mesophyll cells of maize under conditions of natural illumination (I) and in dark followed by light (II).

The changes in the lipid composition of the etioplasts with increasing age, particularly the rapid rise in the GL:PL ratio and the increase in the proportion of MGDG and PG, make it quite clear that etioplasts have a developmental sequence as do chloroplasts in leaves grown under natural light regimes (16).

While it is tempting to correlate these lipid changes solely with the changes occurring in the plastid structure, it should be remembered that Zea mays L. has two different plastid types in the older tissue; the compositional changes may not, therefore, simply reflect changes with respect to age, but may also reflect differences in the lipid composition of the two plastid types and possible different relative numbers of them.

The proportion of linolenic acid (18:3) in the total lipid increases with age both in the isolated plastids and the leaf sections; the changes in the latter are similar to those reported for the equivalent sections of 7-day-old light- and dark-grown maize (14). Calculations of the total fatty acid composition of the leaf sections based on the data reported here (Tables III and V) indicate that there are considerable pools of saturated/unsaturated fatty acids in the tissue which are not associated with those lipids analyzed here, since the calculated proportions of 18:3 are 10 to 15% less than the experimentally determined proportions. Autooxidation of the aliquots taken for TFA analysis can be ruled out since they were taken from the same samples used for the fatty acid analysis of the individual lipids and these undergo more manipulations than do the aliquots for total fatty acid composition, and would therefore be susceptible to oxidation.

The increase in the proportion of 18:3 with age in the total lipid reflects not only the increase in the levels of the galactolipid with which it is preferentially associated, but also an increase in the proportion of 18:3 in those new molecules being synthesized. Calculations show that the new MGDG molecules synthesized between Y and Z contain about 90 mol % 18:3 and is evidence that light is not required for its synthesis.

Although the fatty acid compositions of the light- and dark-grown maize leaves are qualitatively similar, quantitatively there is much less present in the etiolated leaf. This applies particularly to linolenic acid and is a reflection of the much lower galactolipid content of etioplasts (18). Despite the increases in galactolipid with age, the MGDG content of the oldest etiolated section is only 48% of that in the equivalent section of normal light-grown leaves (section C) (14). This means that the number of molecules of 18:3 in the MGDG of section Z is only 56% of that in section C of light-grown leaves. The quantitative increases in 18:3 following illumination (10) are therefore undoubtedly a reflection of the increase in galactolipid content of the tissue (24, 26).

The results reported here suggest that the marked and characteristic increases in both the total amount and the proportion of galactolipid, and hence linolenic acid, in etiolated leaves following their illumination should be greater if young etiolated tissue is used than if old etiolated tissue is used. Roughan and Boardman (22) found little change in the fatty acid composition of MGDG and DGDG molecules isolated from peas (10–11 days old) and beans (11–14 days old) after 24- or 72-hr illumination, respectively, even though there was a 110% increase in MGDG in peas (24 hr) and a 246% increase in MGDG in bean (72 hr). Similarly Bahl et al. (1) found little difference in the lipid or fatty acid composition of envelope membranes isolated from chloroplasts, etiochloroplasts, or etioplasts of wheat. In both of these cases it is possible that the etiolated tissue used was "mature." When changes in the fatty acid composition of individual lipids do occur upon the illumination of the tissue (10, 26) it suggests that that tissue was relatively immature.

The increase in the amount of Pchl(ide) with tissue age suggests that the "greening potential" of the etiolated tissue also increases with age. This suggests the development of other biochemical processes within the plastids beyond those implied from the analyses described here, and which might be expected from the greening studies of Robertson and Laetsch (20). The presence of only a single Pchl(ide) species is contrary to the findings of Horton and Leech (9) using maize, and Klein and Schiff (12) using bean. The former demonstrated two absorption peaks (using a method identical with that described here) in extracts of 7-day-old maize leaves while the latter also found two peaks and that the relative proportions of each altered as the tissue aged. The reason for this difference is not known but again it might be a function of tissue age.

The analyses reported in this paper together with those of Robertson and Laetsch make it quite clear that within an etiolated monocotyledon leaf the etioplast population is heterogeneous with respect to its ultrastructure, composition, and its developmental potential. It is therefore imperative that defined areas of etiolated leaves are used for studies in which the etioplast-chloroplast transformation is being used as a model for studying chloroplast development.

Since the etiolated leaf may be more complex with respect to its composition than it at first appears and since etioplasts are not normally considered to be present in naturally greening leaves (except at the leaf base of a monocotyledon shoot where the light intensity will be low because of the close proximity of the leaves [14]) it would seem that the monocotyledon leaf from a shoot grown under natural diurnal light regimes offers the best way of studying the events surrounding the natural development of chloroplasts from proplastids.

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