CHLOROPLAST DIVISION IN SPINACH LEAVES EXAMINED BY SCANNING ELECTRON MICROSCOPY AND FREEZE-ETCHING

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SUMMARY

Spinach leaf disks were cultured for 5 days in low-intensity green light and then were transferred to high-intensity white light. Harvests over the next 16 h established that cell area increased by about 80%, and chloroplast number per cell increased by about 65%, while the percentage of dumbbell-shaped chloroplasts per cell decreased by 65%. Freeze-etch replicas of fixed and unfixed leaf disks, as well as scanning electron-microscope preparations of fixed material, contained dumbbell-shaped chloroplasts constricted to various degrees. Freeze-etch replicas of unfixed cells from young leaf bases, in which the number of chloroplasts per cell is known to be rapidly increasing, also contained many constricted chloroplasts. It is concluded that dumbbell-shaped chloroplasts occur in vivo and represent a stage in the division of chloroplasts.

INTRODUCTION

During the normal growth of higher plant leaves a massive increase occurs in the chloroplast population. Chloroplasts in large numbers are required for the large increase in the chloroplast population per cell which occurs during cell expansion and to supply chloroplasts to new cells arising by cell division (Possingham, 1980). It has been suggested for a long time, and is now generally accepted, that these organelles increase in number by the division of pre-existing plastids (Schimper, 1885; Kirk & Tilney-Bassett, 1978). In lower plants, a sequence in which 1 chloroplast passes through a dumbbell stage to give 2 daughter chloroplasts has been convincingly documented by cinematography (Green, 1964). In higher plants, however, although chloroplast profiles with central constrictions have been observed in a number of species by light and electron microscopy, evidence that dumbbell-shaped chloroplasts actually divide is largely circumstantial (Lance, 1958; Senser & Schott, 1964; Cran & Possingham, 1972; Esau, 1972; Leech, Rumsby & Thomson, 1973; Whatley, 1974). Nevertheless, in both spinach and tobacco (Boasson & Laetsch, 1969; Possingham & Saurer, 1969) and more recently in wheat (Boffey, Ellis, Sellden & Leech, 1979) and in bean (Whatley, 1980), microscope observations of developing leaf cells have established that there is a correlation between the increase in chloroplast number and the presence of dumbbell-shaped chloroplasts.

In the present work we have applied scanning electron microscopy (SEM) and

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freeze-etching to spinach leaf tissues to obtain more detailed information on the ultrastructure and 3-dimensional appearance of their chloroplasts during division. We examined cells from the base of young spinach leaves at a stage when chloroplast numbers are known to be rapidly increasing (Possingham & Saurer, 1969). We also used the cultured leaf disk system (Possingham & Smith, 1972), in which the proportion of dumbbell-shaped chloroplasts is increased by culturing tissue consecutively at different light intensities.

MATERIALS AND METHODS

Spinach (Spinacea oleracea L.) plants were grown in aerated nutrient solution in a growth cabinet as in earlier experiments (Possingham & Saurer, 1969). Disks were excised from the bases of 2-cm-long spinach leaves and were cultured on nutrient agar plates as previously described (Possingham & Smith, 1972). The plates were kept in continuous green light (60 μE m⁻² s⁻¹) for 5 d and were then transferred to continuous white light (300 μE m⁻² s⁻¹) for 0-24 h.

Light microscopy. Sections approximately 100 μm thick were cut from fresh leaves supported by plastic foam, using a sledge microtome. These were mounted in Honda medium and examined in a Zeiss Photomicroscope III using Nomarski interference or phase-contrast optics. Chloroplast number per cell, chloroplast area and cell area were measured in separated cells of glutaraldehyde-fixed tissues, as previously described (Possingham & Smith, 1972) for 5 experiments. The percentage of dumbbells per cell was also measured for one of these experiments.

Scanning electron microscopy. Disks were fixed in 3 % glutaraldehyde, 0·1 M sodium phosphate buffer for 2-4 h at room temperature, washed in buffer and postfixed in aqueous 1 % osmium tetroxide. Additional osmium was bound to the tissue using thiocarbohydrazide as a ligand (Kelley, Dekker & Bluemink, 1973). After being dehydrated through an acetone series, the specimens were dried from liquid CO₂ in a Balzers Union Critical Point Dryer, and fragmented by cutting or tearing. The tissue pieces were mounted on stubs and examined in a Philips 500 SEM. Some samples were gold-coated in an ISI PS-2 sputter coater before observation.

Freeze-etching. Cultured leaf disks and the bases of leaves less than 2 cm in length, fresh or fixed in 3 % glutaraldehyde, 0·1 M sodium phosphate buffer, were processed in a Balzers freeze-etch unit. Fixed tissue was transferred to 25 % glycerol for a minimum of 4 h before freezing in 25 % glycerol on gold specimen disks in liquid Freon 22 at −150 °C.

Fresh tissue was surrounded by water and frozen as above. Freeze-etch replicas were cleaned by an initial soaking in 80 % sulphuric acid overnight at room temperature followed by 4-5 h at 60 °C. After being rinsed in water, the replicas were transferred to sodium hypochlorite (3·5 % available chlorine) for 4 h, rinsed in distilled water and mounted.

RESULTS

Cell size, chloroplast number and percent dumbbells

When leaf disks cultured for 5 days in low-intensity green light were exposed to high-intensity white light, the area of their cells increased by 80 % over 16 h (Fig. 1). Changes also occurred in the number and conformation of the chloroplasts (Fig. 1). After a lag period of about 8 h, the number of chloroplasts per cell increased by nearly 65 % over the next 5 h. Furthermore, at t₀, approximately 65 % of the chloroplasts were in a dumbbell configuration, whereas after 16 h in white light, only 20 % of the chloroplasts were constricted. At t₁₆, the unconstricted, ovoid chloroplasts frequently occurred in mirror-image pairs.
Light microscopy

Sections of living tissue were cut from disks cultured for 5 days in green light and examined using Nomarski interference optics. Only cells exhibiting protoplasmic streaming were observed and photographed (Figs. 2, 3). The cells contained chloroplasts which were either approximately round or dumbbell-shaped, i.e. oblong and constricted to varying degrees near the centre (Fig. 2). Some chloroplasts were in the mobile or amoeboid phase (Fasse-Fransisket, 1956). These chloroplasts were irregular in outline; pseudopodia-like projections were common and, in some instances, measured several microns in length. Occasionally, 2 chloroplasts appeared to be connected by such projections (arrow, Fig. 3), but careful examination disclosed a small gap between them. The grana in these chloroplasts were not always evenly distributed,
as areas free of grana were often observed at the periphery of the chloroplasts and at the extremities of the dumbbell-shaped organelles (Fig. 2).

Small structures similar in appearance to mitochondria, were also numerous in living cells. They frequently lay clustered near the chloroplasts.

**SEM**

Chloroplasts were located in a single layer close to the cell wall (Fig. 4). They were shaped either like flattened spheroids 1-1.15 μm in diameter or like dumbbells (Figs. 5, 6) measuring 1-1.5 μm by 1.5-2 μm, and were about 0.2-0.3 μm deep. Dumbbell-shaped chloroplasts could be differentiated from pairs of unconnected ovoid chloroplasts (Fig. 4). Although chloroplasts were constricted to varying extents, in most cases they were in an hourglass configuration (Fig. 5). Some chloroplasts showed a distinct neck of variable diameter connecting the 2 halves of the dumbbell (Fig. 6).

Organelles similar in shape and size to mitochondria were frequently observed adjacent to chloroplasts (Fig. 4). In some instances, they appeared to lie in the groove formed by the constriction in a dumbbell.

**Freeze-fracture and freeze-etch**

Chloroplast shape varied considerably. Chloroplast profiles were circular, lenticular or oblong in shape (Figs. 7-16). Some oblong chloroplasts showed only a slight indentation near the middle (Fig. 10). Others were more markedly pinched in at the centre, forming a dumbbell-shape. Asymmetrical constrictions were observed occasionally. Whereas in most instances chloroplasts were the shape of an hourglass (Figs. 11, 12, 13), occasionally a tube-like bridge of uniform diameter was seen between the 2 postulated daughter chloroplasts (Figs. 13, 14). Occasionally, dumbbell-shaped chloro-
Figs. 9–16. Freeze-etching of fixed leaf disks.

Fig. 9. Part of cell showing the envelope surface of 3 chloroplasts (2 dumbbells, 1 ovoid) and the stroma and thylakoids (arrow) of a fourth. × 14,800.

Fig. 10. Oblong chloroplast with a slight indentation near the centre. The unevenness of the surface suggests an irregularity of outline as described in Fig. 2. × 11,600.

Fig. 11. Hourglass-shaped chloroplast clearly showing the continuity of the surface membranes from one half to the other. × 16,400.
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Plants with surface irregularities similar to small pseudopodia-like projections seen in living cells (Figs. 2, 3) were observed in the freeze-etch preparations (Fig. 14).

The planes of fracture occurred so as to show the envelope (Figs. 7, 9–11, 13, 14) or the stroma (Figs. 7, 9, 12, 15, 16). The chloroplasts were most commonly fractured in a plane perpendicular to that of the thylakoid sheets (Figs. 7, 15, 16); occasionally, however, the fracture exposed large areas of thylakoid membrane as it passed through the organelle in the same plane as the thylakoids (Fig. 12).

The system of thylakoids appeared continuous throughout the length of the dumbbell-shaped profiles. Membranes frequently lay within the narrowed portion of the dumbbell (Figs. 12, 15) and at no point during division were 2 ‘daughter’ systems evident.

Dumbbell-shaped chloroplasts were also observed in replicas of unfixed leaf disks (Fig. 7) and of unfixed young leaves (Fig. 8).

Discussion

Despite the lack of direct evidence, such as has been provided by cinematography for lower plants (Green, 1964), good indirect evidence is now available that there is a precursor–product relationship between dumbbell-shaped chloroplasts and new chloroplasts. In the present experiment with leaf disks, the number of chloroplasts per cell increased by nearly 65% within 12 h. Over the same period, the proportion of dumbbell-shaped chloroplasts decreased by 65%. It could perhaps be argued that the dumbbell represents a change in chloroplast shape induced by green light and that it is not strictly a division figure. The present results suggest that this is unlikely, as a correlation exists between the timing and extent of the increase in plastid number and the decrease in the percentage of dumbbells. Furthermore, electron-microscope observation of these disks provides no evidence of proplastids, etioplasts or other plastid forms that might represent an alternative pathway for chloroplast formation.

The large size of the chloroplast makes it almost impossible to focus clearly on the entire organelle in the light microscope, particularly in the area of the constriction. With SEM or with transmission electron microscopy of freeze-etch replicas it is possible, however, to distinguish between constricting chloroplasts and shapes that form when chloroplasts overlap or emit pseudopodia-like structures. In the present work, we have demonstrated that chloroplasts with dumbbell configurations exist in 2 tissues where chloroplast numbers are known to be increasing rapidly, i.e. cultured spinach leaf disks and young spinach leaves. Many constricted chloroplasts were observed in SEM preparations of fixed material and in freeze-etch replicas of fixed and unfixed leaf and leaf disk tissue. Taken in conjunction with the data presented in Figs. 1–3, we believe these observations demonstrate unmistakably that dumbbell-shaped chloroplasts exist in vivo and represent a stage in the division of chloroplasts.

It is interesting to note at this stage the considerable similarity in the conformation of isolated and intracellularly located dumbbells, even to the presence of pseudopodia-like projections, when observed by Nomarski interference-contrast microscopy, SEM and freeze-etching.
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We have observed dumbbells constricted to various degrees, suggesting a division sequence from round plastid to elongated cylinder to slightly indented cylinder, and then passing either directly to an almost complete constriction or, more rarely, passing through a stage with a tube-like connection between the 2 daughter plastids. Somewhat similar constriction figures have been observed previously with the light microscope (Ridley & Leech, 1970; Possingham, 1976; Vujicic & Possingham, unpublished; see also Kameya & Takahashi, 1971).

The mechanism by which the constriction develops is unknown. As in mature chloroplasts of *Agapanthus* (Fasse-Fransisket, 1956), the constriction was generally centrally located. Both SEM and freeze-etch preparations suggest that the constriction encircles the chloroplast, as though formed by the tightening of a drawstring. The freeze-etch planes of fracture lay parallel or perpendicular to the thylakoid sheets. Dumbbell-shaped chloroplasts with both orientations of thylakoids were observed, suggesting that the constriction occurs both in the breadth and the thickness of the organelle, i.e. encircles the chloroplast. No structures either inside the plastid, such as microtubules, or outside in the cytoplasm, such as attachment fibres, could be responsible for generating the constriction.

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REFERENCES


Fig. 12. Hourglass-shaped chloroplast fractured in the plane of the thylakoids (t). These lie throughout the organelle, even in the area of the constriction. ×17,200.

Fig. 13. Constricted chloroplast with tube-like bridge connecting the 2 sides. A mitochondrion (m) lies in the cytoplasm between the neck of the dumbbell and another chloroplast (c). ×19,200.

Fig. 14. Constricted chloroplast as in Figure 13, but with a pseudopodium-like projection (p) at one end. ×13,000.

Fig. 15. Hourglass-shaped chloroplast fractured in a plane perpendicular to that of the thylakoids (arrows). These extend longitudinally throughout the profile, and are not interrupted in the constricted area. ×14,000.

Fig. 16. Constricted chloroplast with a central tube-like bridge. The plane of fracture has disclosed the stroma and thylakoids in part of the organelle, showing that they extend longitudinally. ×21,200.


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