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Short Communication

Chloroplast Structure and Function in Tissue Cultures of a C_4 Plant[1]

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The initial determination that C_4-dicarboxylic acids were the primary photosynthetic products in a higher plant was made by Kortschak *et al.* (6). This photosynthetic carbon-fixation pathway is now known to occur in many angiosperm taxa, and specific aspects of the pathway have received considerable attention (5). The leaves of C_4 plants have a characteristic anatomy consisting of concentric bundle sheath and mesophyll cell layers around the vascular bundles. These two distinct cell layers possess chloroplasts which have either structural or size dimorphism. The bundle sheath cell chloroplasts function as amyloplasts (9, 10).

It has been proposed that the primary carboxylation yielding C_4-dicarboxylic acids takes place in the mesophyll cell chloroplasts, and that these products are transported to the bundle sheath cell chloroplasts where decarboxylation and subsequent refixation via the Calvin Cycle takes place (3, 5, 9). This scheme necessitates a causal relationship between leaf and chloroplast structure and photosynthetic carboxylation in C_4 plants. This assumption can be tested by investigating carbon-fixation in tissue cultures of C_4 plants, because tissue cultures lack the structural features associated with leaves of C_4 plants.

Froelichia gracilis (Hook.) Moq. is a member of the Amaranthaceae, and its leaves possess all the structural features associated with C_4 plants (10). It has also been shown to have the low CO_2 compensation point characteristic of C_4 species (15).

MATERIALS AND METHODS

Tissue cultures of *F. gracilis* were obtained from stem explants of mature plants. Cultures were initiated and maintained on a modified Murashige and Skoog medium (11). The medium contained kinetin, 1 mg/l, naphthalene acetic acid, 0.5 mg/l, sucrose, 20 g/l, and agar, 8 g/l. The tissue was maintained at 25 C under continuous light of 700 ft-c provided by Sylvania VHO fluorescent tubes. The strain of callus used in this experiment has been in culture for a year and a half.

Tissue was prepared for examination by light and electron microscopy by fixing in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.8, for 3 hr at room temperature. The fixation medium contained $CaCl_2$ at 20 μg/ml glutaraldehyde buffer solution. Tissue was rinsed three times in buffer at 4 C for a total of 60 min. Tissue was postfixed with 2% (w/v) OsO_4 in 0.1 M sodium cacodylate buffer for 1 to 2 hr at 4 C. Dehydration was accomplished with a graded acetone series at 4 C. Tissue was placed in 1.0% (w/v) uranium nitrate in 70% (v/v) acetone overnight at 4 C. The acetone series was terminated with one change of 100% (v/v) acetone at 4 C for 20 min and two changes of 30 min each at room temperature. The tissue was transferred to propylene oxide and then to a graded propylene oxide and Epon series (2). The Epon was kept at room temperature for 24 hr and then incubated at 60 C for 2 days and 95 C for 1 day. Sections were cut with a diamond knife, placed on uncoated grids, stained with lead (13), and examined with a Zeiss EM 9A microscope.

Table I. *Distribution of Radioactivity from $^{14}CO_2$ Fixed Tissue Cultured from F. gracilis*

After removal of the bulk of the agar, the callus was placed in a 100-ml beaker with 2 ml of water. Tungsten light, through water to prevent heating, gave 2000 ft-c at the position of the sample. The temperature in the beaker remained at 25 C throughout the experiment. After 20 min of preillumination, the beaker was covered with Saran Wrap and 100 μc (0.18 mg) $^{14}CO_2$ was injected, giving a $^{14}CO_2$ concentration of 0.7%. At 8 sec the sample was removed from the beaker, dropped into boiling 95% (v/v) ethanol, and boiled for 5 min. The tissue was extracted in a Waring Blendor with 95% (v/v) ethanol, filtered, and extracted with water. Aliquots of the extracts and the insoluble residue were dried and counted on planchets with a Nuclear Equipment Model E counter (1 μc = 30,000 counts). Over 99% of the activity appeared in the extracts. Aliquots of the extracts containing about 3000 counts were spotted on Whatman No. 1 paper and chromatographed with 70% (v/v) ethanol. Known radioactive glycerate-3-P, aspartate and malate were run at the same time. Spots identified as glycerate-3-P or sugar phosphate were eluted, hydrolyzed with alkaline phosphatase, and rechromatographed to confirm the identification. Chromatograms were counted on a Packard Chromatogram Scanner.

	R_F	Total Counts
		%
Glycerate-3-P	0.02	4
Sugar phosphates	0.19	25
Aspartate	0.36	26
Malate	0.50	27
Unknown	0.58	16
Glycerate	0.67	1

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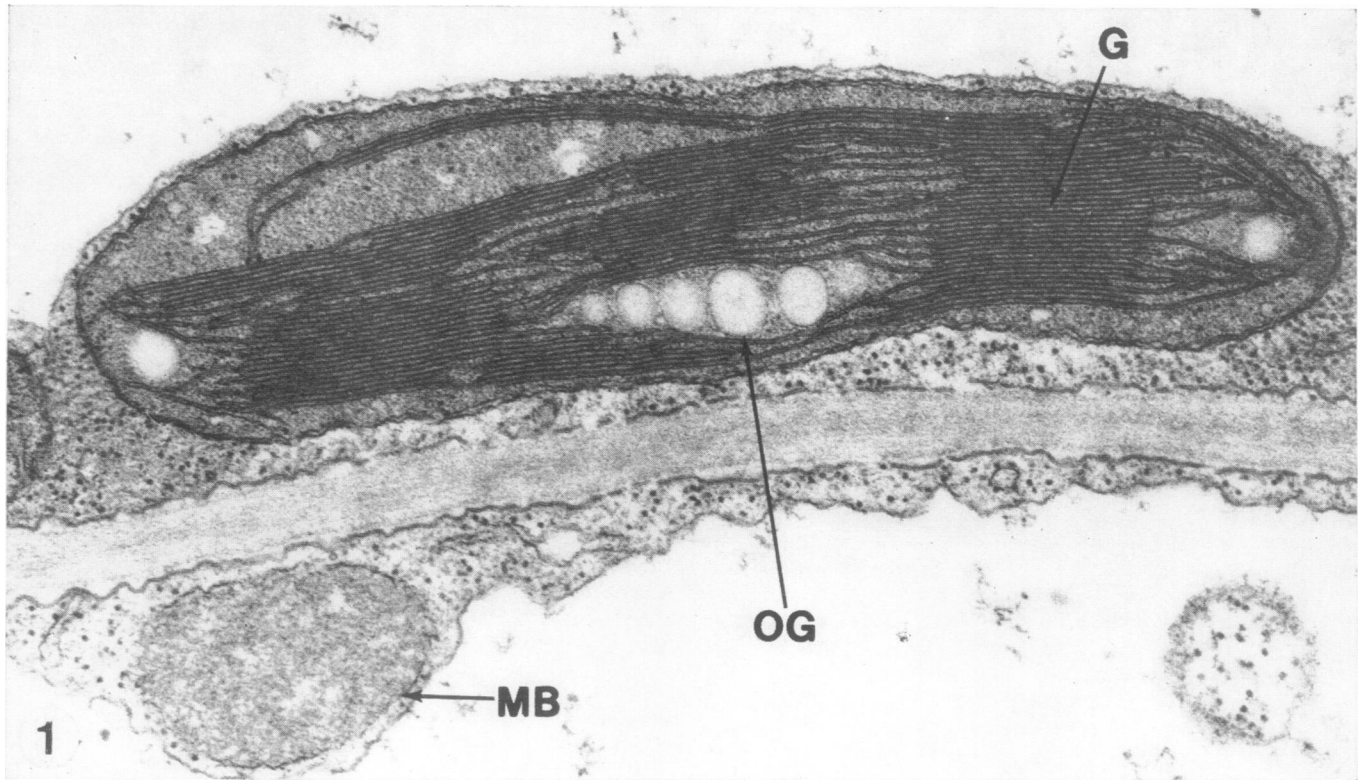

FIG. 1. Chloroplast in cell of *F. gracilis* callus. Grana (G) are well developed and osmiophilic globules (OG) are numerous. Microbodies (MB) are similar to those found in leaves. × 35,750.

RESULTS AND DISCUSSION

The total uptake of $^{14}CO_2$ was 10,800 counts (a dark control had 530 counts); with a residue weight of 25 mg this is equivalent to a rate of 5 mg CO_2/g of residue·hr, about $\frac{1}{10}$ of usual values for leaves of C_4 plants. A similar difference in the photosynthetic $^{14}CO_2$ incorporation in tobacco callus and leaves has been reported (11). The low proportion of activity in glycerate-3-P, and high activity in 4-carbon acids (Table I), is typical of the C_4 pathway.

The callus has a fairly typical anatomy. Large parenchyma cells containing chloroplasts make up most of the tissue. Islands of meristematic cells and small clusters of lignified cells are randomly scattered throughout the callus. A typical chloroplast in a callus cell is illustrated in Figure 1. They are characterized by well developed grana and are similar to the chloroplasts of the mesophyll cells of *F. gracilis* leaves. Many of the callus chloroplasts have unusually large grana. Starch is also found in chloroplasts in callus cells. The peripheral reticulum is poorly developed and it is not present in chloroplasts in Figure 1. The general cytological features of the chloroplast-containing cells are similar to those of the chlorenchyma cells in the leaves. This includes the presence of peroxisome-like microbodies.

The carbon-fixation pattern in tissue cultures of *F. gracilis* is typical of C_4 plants. This pattern contrasts strikingly with the C_3 plant pattern obtained with green parenchyma excised from sugar cane stems (7). The callus induced from the latter tissue does not synthesize chlorophyll, so direct comparison with *F. gracilis* callus cannot be made. Only one type of chloroplast-containing cell is found in this tissue, and all the chloroplasts in these cells have well developed grana. Starch is synthesized and the $^{14}CO_2$ incorporation data show that glycerate-3-P and sugar phosphates are produced in these chloroplasts. This demonstrates that all elements of the C_4 and C_3 photosynthetic carbon-fixation pathways are present in these cells. Structural specialization of cells and/or chloroplasts is not causally related to the C_4 pathway. These results suggest that both carbon-fixation pathways could take place in the mesophyll cells of C_4 plants. Photosynthetic intermediates would then be transported to the functional amyloplasts in the bundle sheath cells. This hypothesis is supported by Kortschak's evidence on sucrose synthesis in sugar cane leaves (8) and by the recent work of Baldry *et al.* (1).

The chloroplasts of *F. gracilis* callus do not have a well defined peripheral reticulum even though this membrane system is highly developed in the mesophyll cell chloroplasts of this species. Since the mesophyll cells in the leaf export photosynthetic products to the bundle sheath cells, there is bound to be a very active interchange of materials between the cytoplasm and chloroplasts in mesophyll cells. The peripheral reticulum greatly increases the surface area of the inner membrane of the chloroplast envelope, and its involvement in the transport of materials across the envelope has been suggested (10). If the outer membrane were permeable and the inner membrane selectively permeable, as appears to be the case in mitochondria (12), and hence limiting to rapid transport across the envelope, a great increase of the limiting layer would be a logical way to facilitate transport. There is no compartmentalization of function in the cells of the callus, so the directed transport of photosynthates would not be expected. This would result in less transport across the chloroplast envelope and might provide a rationale for the restricted development of the peripheral reticulum.

What is the role of the specialized leaf anatomy in C_4 plants, since these results show that carbon-fixation pathways are not related to leaf and chloroplast structure? It was suggested some time ago that the concentric chlorenchyma provides a

mechanism to re-assimilate CO_2 if most photorespiration occurs in the bundle sheath cells (9). The distribution of peroxisomes and mitochondria in the two cell layers (4, 10) and the known localization of enzymes involved in photorespiration (14) make this interpretation increasingly valid. The ability to retain assimilated CO_2 is the most important factor in the high growth rates exhibited by C_4 plants, and this important adaptive feature appears to be a function of the specialized leaf anatomy.

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