LOCALIZATION OF ACTIN FILAMENTS IN INTERNODAL CELLS OF CHARACEAN ALGAE

A Scanning and Transmission Electron Microscope Study

YOLANDE M. KERSEY and NORMAN K. WESSELLS

From the Department of Biological Sciences, Stanford University, Stanford, California 94305. Dr. Kersey's present address is the Cardiovascular Research Institute, University of California at San Francisco, San Francisco, California 94143.

ABSTRACT

New methods of visualizing subcortical actin filament bundles, or *fibrils*, in Characean internodes confirm that they are associated with chloroplasts at the surface facing the streaming endoplasm, and reveal that they are continuous over long distances. With the scanning electron microscope, an average of four to six fibrils are seen bridging a file of chloroplasts. The same configuration appears in negatively stained preparations of large blocks of chloroplast files connected by actin fibrils. Few branches of the subcortical fibrils are evident. These findings are discussed with respect to the mechanism of cytoplasmic streaming in Characeae.

Cytoplasmic streaming in green algae and higher plants occurs along microscopically visible "fibrils" (7, 15, 18, 21, 22, 26, 35), resolvable at the electron microscope level into bundles of ca. 5- to 7-nm microfilaments (6, 7, 20, 21, 22, 25, 27–31, These filaments combine reversibly with heavy meromyosin (HMM) from rabbit muscle (7, 21, 22, 28, 34), as does muscle actin.

Elongate microfilament bundles of roughly the same dimensions (ca. 0.1–0.2 μm) as the subcortical fibrils in Characean cells are also found in a variety of animal cells, where immunofluorescence techniques have shown that they contain actin, myosin, and tropomyosin (23, 24, 33). In green plants, the actin filaments composing the bundles are apparently all of the same polarity, as evidenced by binding of HMM from rabbit muscle (7, 21, 22, 28, 29). In Characean algae, the polarity of the actin filaments is such that HMM arrowheads point in the direction opposite to that of the cytoplasmic stream (21, 22). Thus the direction of

force generation with respect to HMM arrowheads is the same in these algae as in the striated muscle sarcomere (14). A similar relationship also seems to exist in the mitotic spindles of locust testis cells, where the majority of HMM arrowheads point toward the metaphase plate (11). This direction is opposite to that of cytoplasmic particle movement described in *Haemanthus* endosperm cells at metaphase (2, 3). Therefore, by analogy with striated muscle, Gawadi (11) postulated that actin filaments are involved in particle movement toward the spindle poles. Actin is known to exist in the mitotic spindles of *Haemanthus* endosperm (9), as well as in the spindles of various dividing animal cells (8, 10, 11, 13). It seems likely, therefore, that actin functions similarly in the two kingdoms.

Although it is now generally conceded that the fibrils visible at the light microscope level participate in generation of the motive force for streaming in Characean algae, the mechanism is still unclear. Jarosch, who was the first to observe

motile fibrils in isolated cytoplasmic droplets, advanced a "screw-mechanical" theory by which torsional movements of the microfilament bundles propel the cytoplasmic matrix (16). Recently, Allen (1) proposed that immobile subcortical fibrils attached to the chloroplasts possess branches that extend into the endoplasm, where they undulate like sperm tails to propel the streaming cytoplasm. In another recent report, published after the current research was completed, Williamson (35) found that, in cell models, cytoplasmic particles move along the subcortical fibrils in the presence of ATP, but remain immobile and attached to the fibrils in its absence. Pyrophosphate does not elicit movement but does detach particles from the fibrils. From these observations, Williamson postulated that a myosin-like enzyme is attached to the moving particles. He observed no undulations of the fibrils, confirming previous findings of Kamitsubo (18) who examined cytoplasmic movement along fibrils in living plant cells.

Obviously a comprehensive understanding of the streaming process must include localization of the fibrils associated with motility and knowledge of the relationship between these fibrils and other cellular constituents. To further elucidate the nature of the subcortical fibrils in Characean internodes, we have examined the interior of internodal cells with the scanning electron microscope (SEM) and we have correlated these findings with transmission electron microscopy (TEM) of negatively stained, large blocks of cortical chloroplasts and attached actin fibrils.

MATERIALS AND METHODS

Chara australis and *Nitella flexilis* plants were grown in the greenhouse in partial shade, by rooting in plastic wastebaskets filled to a height of 2 cm with sterile soil and to 30 cm with deionized water. Cultures 5–6-wk old were used in these experiments.

Characean internodes are large cylindrical cells containing a huge central vacuole. Beneath the cell wall is a stationary cortical layer in which rows of chloroplasts follow a steep helical path around the long axis of the cell. The endoplasm streams in a continuous belt around the central vacuole, parallel to the chloroplast rows. A chloroplast-free "indifferent zone" separates apically and basipetally directed cytoplasmic streams.

Negative staining of large blocks of oriented chloroplasts and associated actin filaments from Characean internodes was accomplished as described elsewhere (21, 22). Briefly, a cylinder of about 2–3 mm in length was

excised from a 2–3-cm long internode of *N. flexilis* or *C. australis*, then cut longitudinally adjacent to the clear "indifferent" zone separating the apical and basipetal cytoplasmic streams (see Fig. 1). Generally, most of the cytoplasm was washed away during this process. The plant segment, consisting mainly of cell wall with subjacent chloroplasts and associated actin filaments, was placed cell wall upward in a drop of 0.1 M KCl on the surface of a Formvar and carbon-coated grid, and the cell wall tapped lightly with forceps before being lifted off. The preparation was then rinsed with KCl and stained with 1% uranyl acetate. Samples were examined and photographed in a Hitachi HU 11E electron microscope operated at 75 kV.

SEM was performed on whole cells of *C. australis* fixed in 2% glutaraldehyde and 0.005 M phosphate buffer, pH 7, for 3 h at room temperature, followed by a rinse in the same buffer and postfixation in unbuffered 1% OsO_4 for 30 min at room temperature. Cells were then subjected to freeze drying or critical point drying. In the first procedure, cells were frozen in liquid nitrogen and lyophilized overnight in a Virtis apparatus. For critical point drying, tissues were dehydrated in a graded acetone series and dried in CO_2 in a Sorvall apparatus according to the manufacturer's instructions (DuPont Instruments, Sorvall Operations, Newtown, Conn.). Dried segments excised from cells were cut longitudinally (see Fig. 1) and mounted onto stubs with double-sided sticky tape, and then coated with a ca. 7.5–10-nm layer of gold-palladium, using a Denton evaporator equipped with a rotary-tilt shadowing device. Specimens were examined in a Coates and Welter field emission SEM with and without a high resolution photographic monitor. Photographs were taken on Polaroid Type 52 film.

RESULTS

Negative Staining

Samples prepared for negative staining as shown in Fig. 1 and examined at low magnifications with the TEM reveal rows of chloroplasts bridged by fibrils (Fig. 2). At high magnifications, each fibril is seen to consist of a bundle of filaments capable of forming HMM arrowhead complexes removable with ATP (21, 22). The filaments are of roughly the same diameter (ca. 5–7 nm) and axial repeat (ca. 380 nm) as muscle actin. On the average, 12–15 filaments are visible as one views the upper surface of a negatively stained fibril; this number is what might be expected from filament counts made from TEM photographs of cross sections of fibrils (6, 20, 25, Wessells, unpublished TEM photographs). There are usually four to six fibrils per chloroplast file, just as in thin sections. Therefore, we conclude that these
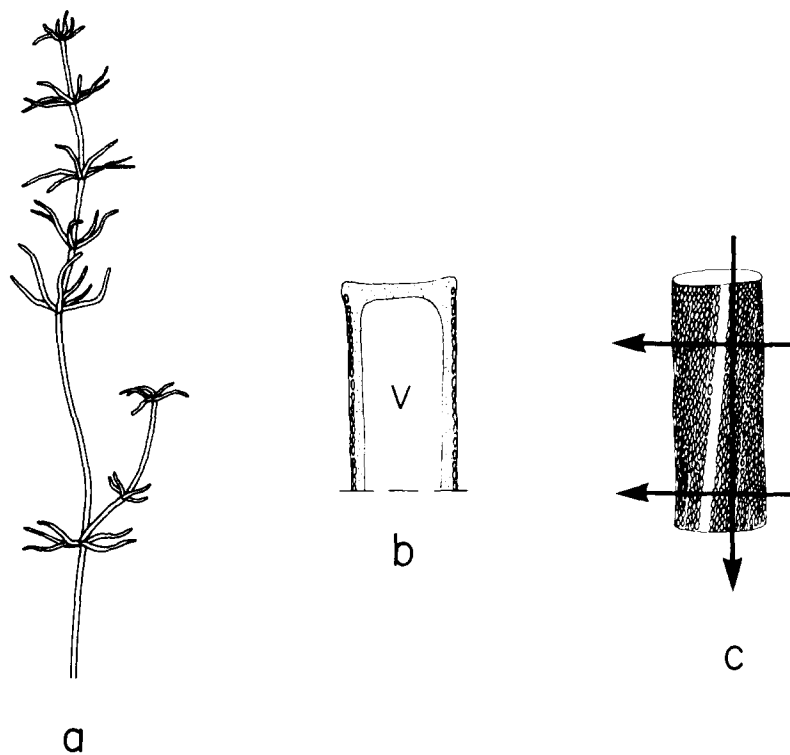
FIGURE 1 (a) Shoot apex of C. *australis*, actual size. Elongate internodes consist of a single multinucleate cell. (b) Cross section of part of an internodal cell. Chloroplasts lie adjacent to the cell wall in the stationary ectoplasm. The cytoplasm streams in a spiral path up one side of the cell and down the other around a large central vacuole (v). (c) Part of an internodal cell. Segments of cell wall and subjacent tissue excised as shown are prepared and examined as described in the text.

fibrils correspond to those first observed in thin sections by Nagai and Rebhun (25).

Although some fibrils are apparently lost during rinsing and negative staining procedures, it is important that in the many preparations examined the number of actin filament bundles per chloroplast file seldom exceeded five and in no instance was greater than six. This suggests that in normal cells there may be little branching of the subcortical fibrils such as that observed by Allen (1) in chloroplast-free areas of "windowed" cells formed by the technique of Kamitsubo (17).

Some negatively stained fibrils appear to be split and frayed, but for the most part this seems to be due to disruption caused by the preparative procedure, since it occurs to a much greater extent near the edges rather than in the center of patches of aligned chloroplasts linked by fibrils. The negatively stained fibrils usually do not overlap and can often be seen to enter and leave the dense areas representing chloroplasts in approximately

straight lines, as seen in Fig. 2, although regularity in placement of the fibrils is not as evident in the figure as at higher magnifications. (At low magnifications uneven stain density results in high contrast which makes good photographic rendition of this material difficult.) Occasionally, a chloroplast row veers off at a sharp angle from the axis of the other chloroplast files. In such instances, generally four or five fibrils are visible connecting the displaced chloroplasts. Since the fibrils retain their normal spacing relative to one another, it appears that they are attached in some manner to the chloroplast surface. Now and then a fibril appears to have pulled away from a chloroplast, remaining connected to it by a fibrous strand (Fig. 2, arrow). Upon examination at high magnification, such strands appear to consist of membranous material, but actin filaments are often evident, which also suggests chloroplast-fibril attachment. Further evidence of such fibril attachment is found in isolated protoplasmic droplets, where

chains of chloroplasts move rapidly through the ground plasm (19, 20). Actin fibrils apparently serve to link the chloroplasts together. The nature of the chloroplast-actin attachment is not known. In TEM photographs of thin sections, Palevitz and Hepler (29) noted thickenings of the chloroplast membrane in areas which might consist of actin fibril attachment points. In the present study, no indication of specialized attachment sites was visible with the SEM, but this might be due to the limited resolution available with this technique.

In a very few negatively stained preparations, the normal groups of ca. four to six fibrils connecting two chloroplasts were aggregated laterally into one or two thick bundles when observed at low magnification, while the usual four to six bundles were discernible at high magnifications (Fig. 3 *a*, *b*). Jarosch (15) observed chloroplasts joined by single thick fibers in light microscope observations on necrotic cells of *N. stelligera*, and similar fibers were seen by Kamitsubo (18) in examining the region adjacent to the "indifferent
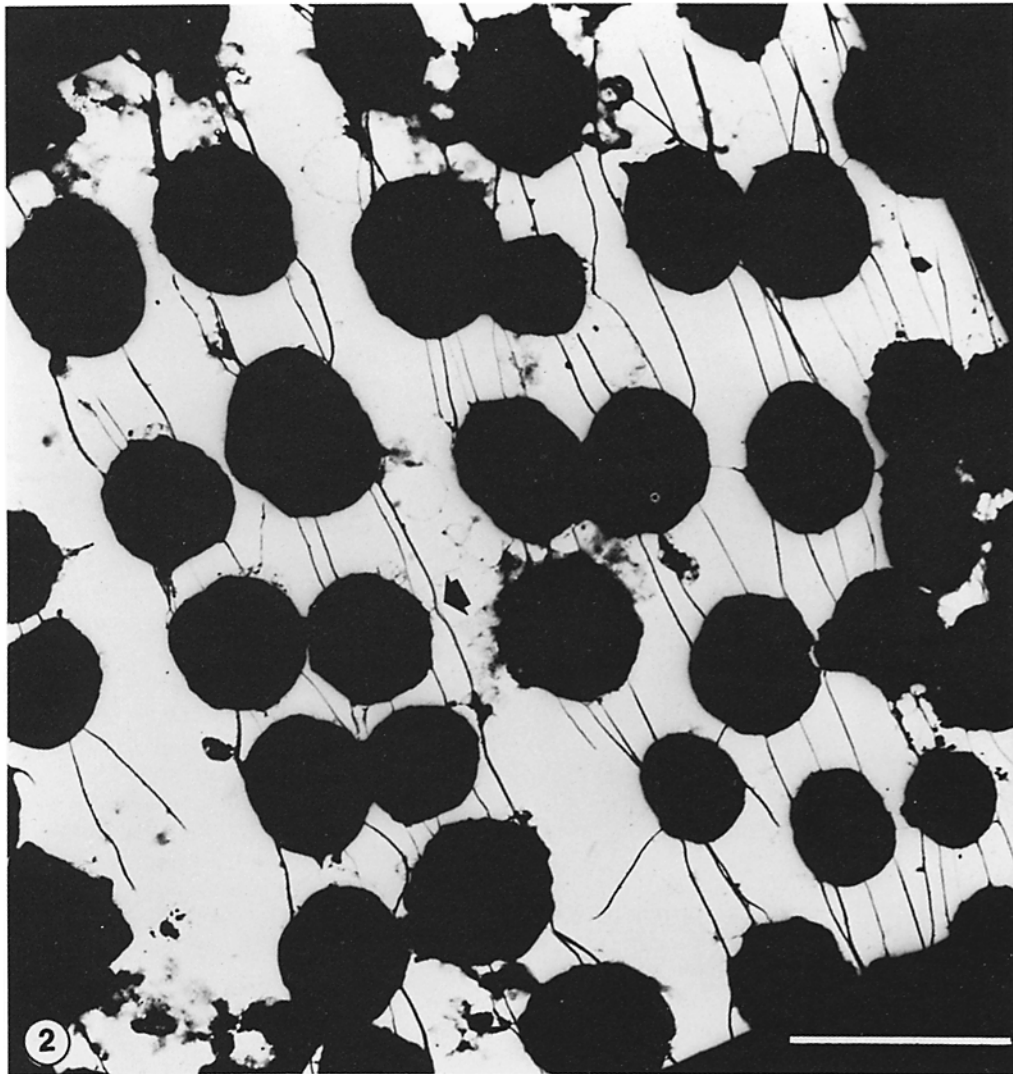

FIGURE 2 Rows of chloroplasts and attached actin fibrils obtained from the cortex of *N. flexilis* and negatively stained as described in the text. Fibrils removed from chloroplasts often remain connected by a fine thread consisting of actin, or membranous material, or both (arrow). Scale = 10 μm; × 2,900.

FIGURE 3 (a) Low power TEM photograph of negatively stained chloroplast rows joined by thick fibers. Scale = 10 μm, × 750. (b) High magnification of thick fibers as in Fig. 3 a reveals that they consist of laterally aggregated 0.1–0.2-μm actin fibrils. Scale = 0.1 μm; × 175,000.

zone" separating apical and basipetal cytoplasmic streams in *Lamprothamnium succinctum*. It may be that these single fibers were also formed by lateral aggregation of fibrils.

In addition, negatively stained preparations reveal small spherical or ellipsoidal particles attached to the actin fibrils. As judged from the contents of thin sections (4, 6, 20, 25, 31), in most instances these particles represent mitochondria, spherosomes, or crystalline or amorphous protein vesicles. Mitochondria have often been seen in association with microfilament bundles in plant cells (see, for example, references 20 and 27) where they are in an advantageous position to contribute ATP believed to be the energy source for cytoplasmic movement (19, 35).

Scanning Electron Microscopy

Scanning electron micrographs also reveal parallel fibrils spanning the chloroplasts in both freeze-dried and critical point-dried material (Figs. 4–10).

In freeze-dried tissue (Figs. 4, 6 a, b, 7, and 8), fibrils occur in parallel strands of indefinite length. We have followed the course of a single fibril up to ca. 200 μm and it may be that some fibrils complete the circuit around the cell. This possibility was previously suggested by O'Brien and McCully (26) concerning cells of *Heracleum mantegazzianum*, where they traced fibrils associated with cytoplasmic motility for 700–800 μm using high resolution light microscopy.

In some areas of freeze-dried tissue, it appears as though a layer of membrane is present over the ectoplasm (Fig. 8). Both rough endoplasmic reticulum and tonoplast are in positions where they could form this membranous layer during preparative steps for scanning microscopy (6, 20, 25, 31). Numerous openings of various sizes mark the membrane surface; some of these could be preparation artifacts. No fibrils are evident on the upper surface of the membrane facing the vacuole, but chloroplasts and aligned fibrils are discernible as elevations in the membrane. The size and spacing of the fibrils beneath the membrane is similar to that of the naked actin fibrils seen where the membrane is absent. In naked areas, short lengths of fibrous material occasionally extend from the parallel fibrils. It is not known whether these short segments represent branches of actin fibrils present in vivo, but this does not seem likely since none are visible in membrane-covered areas. As judged from the appearance of negatively stained preparations, some of these short segments may consist of membrane fragments. In membrane-covered areas, the fibrils appear taut, and assume the same
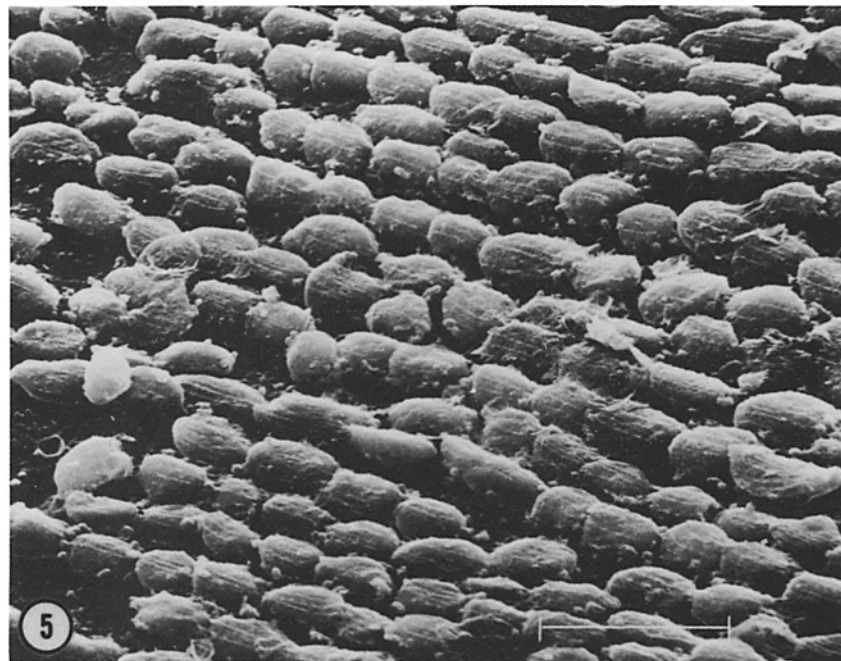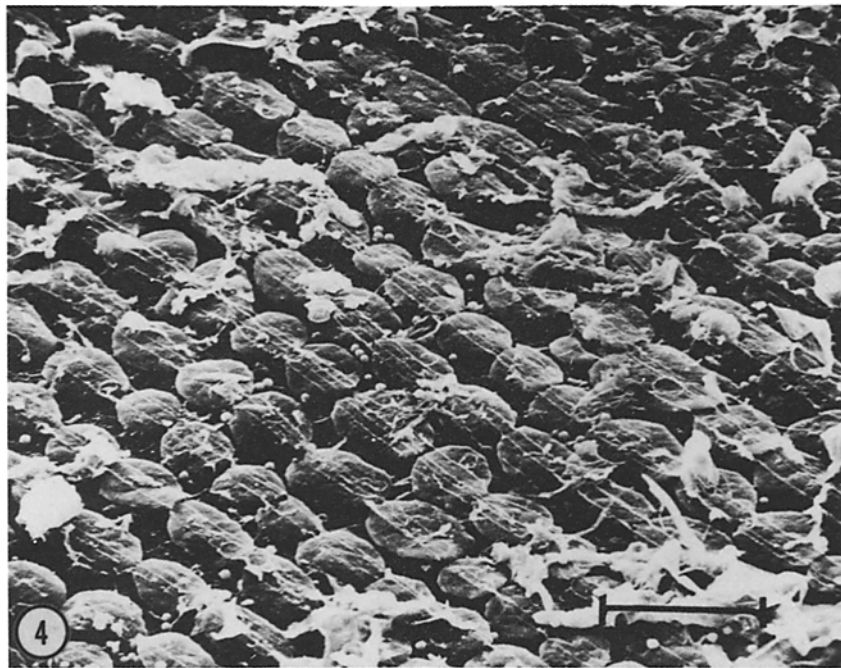
FIGURE 4   SEM of rows of chloroplasts and aligned actin filaments from the cortex of *C. australis*, viewed from the side facing the streaming cytoplasm and central vacuole. Prepared by freeze drying of fixed tissue. Chloroplasts appear collapsed, which may be an artifact of this type of preparation. Scale = 10 μm; × 2,200.

FIGURE 5   SEM of cortex of *C. australis*. Sample prepared by critical point drying. Surprisingly, fibrils are not so well preserved as by freeze drying. Scale = 10 μm; × 2,500.
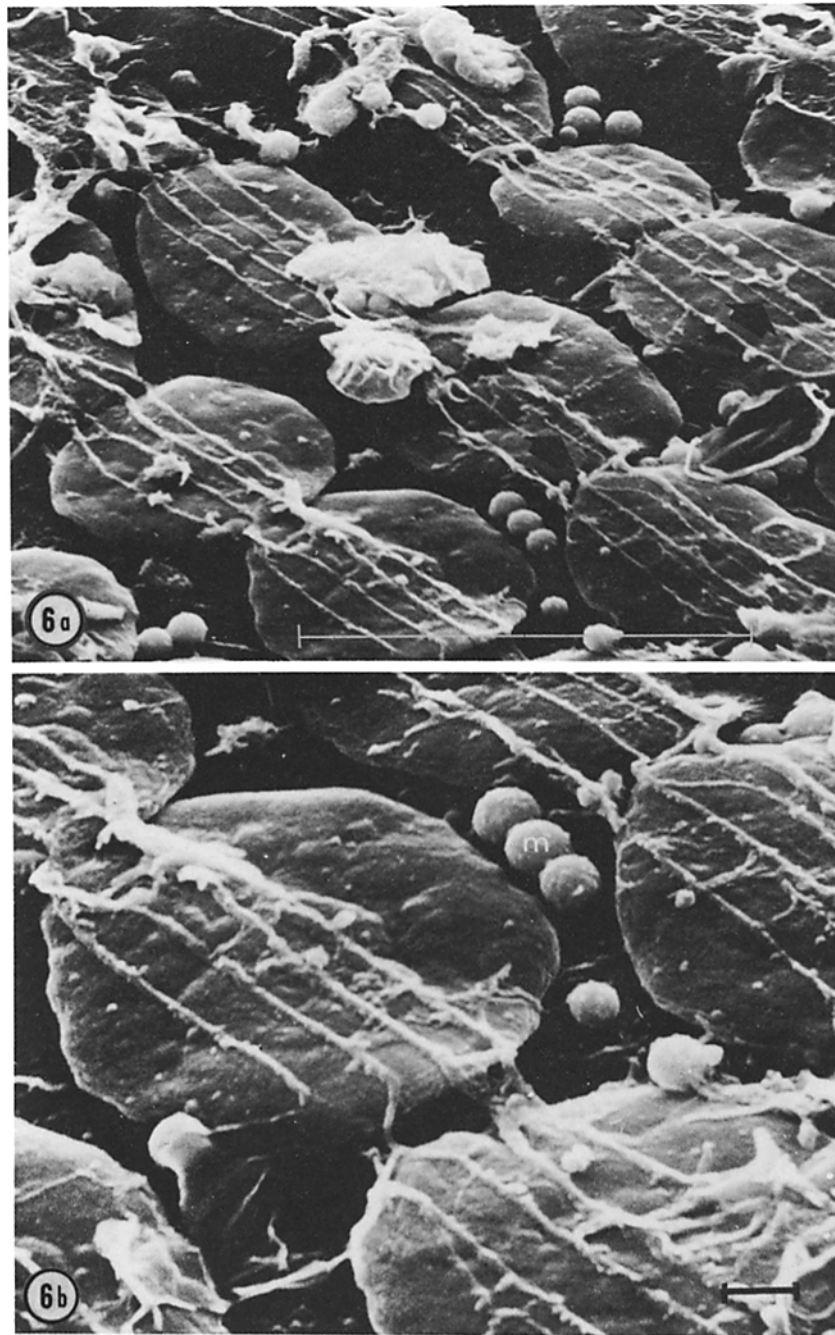
FIGURE 6 (a) SEM of chloroplasts and aligned actin filaments from *C. australis* prepared by freeze-dry technique. Some fibrils—usually only one per chloroplast row—appear thinner than others (arrow). Patches of crumpled membrane, either tonoplast or endoplasmic reticulum, are evident. Scale = 10 µm, × 6,000. (b) Higher magnification of area at bottom center of Fig. 5 a. In most instances, the small spherical particles (m) probably represent mitochondria, or spherosomes, or membrane-bound crystalline or amorphous protein vesicles. Fibrils appear slack between chloroplasts in a file. Scale = 1 µm, × 10,500.
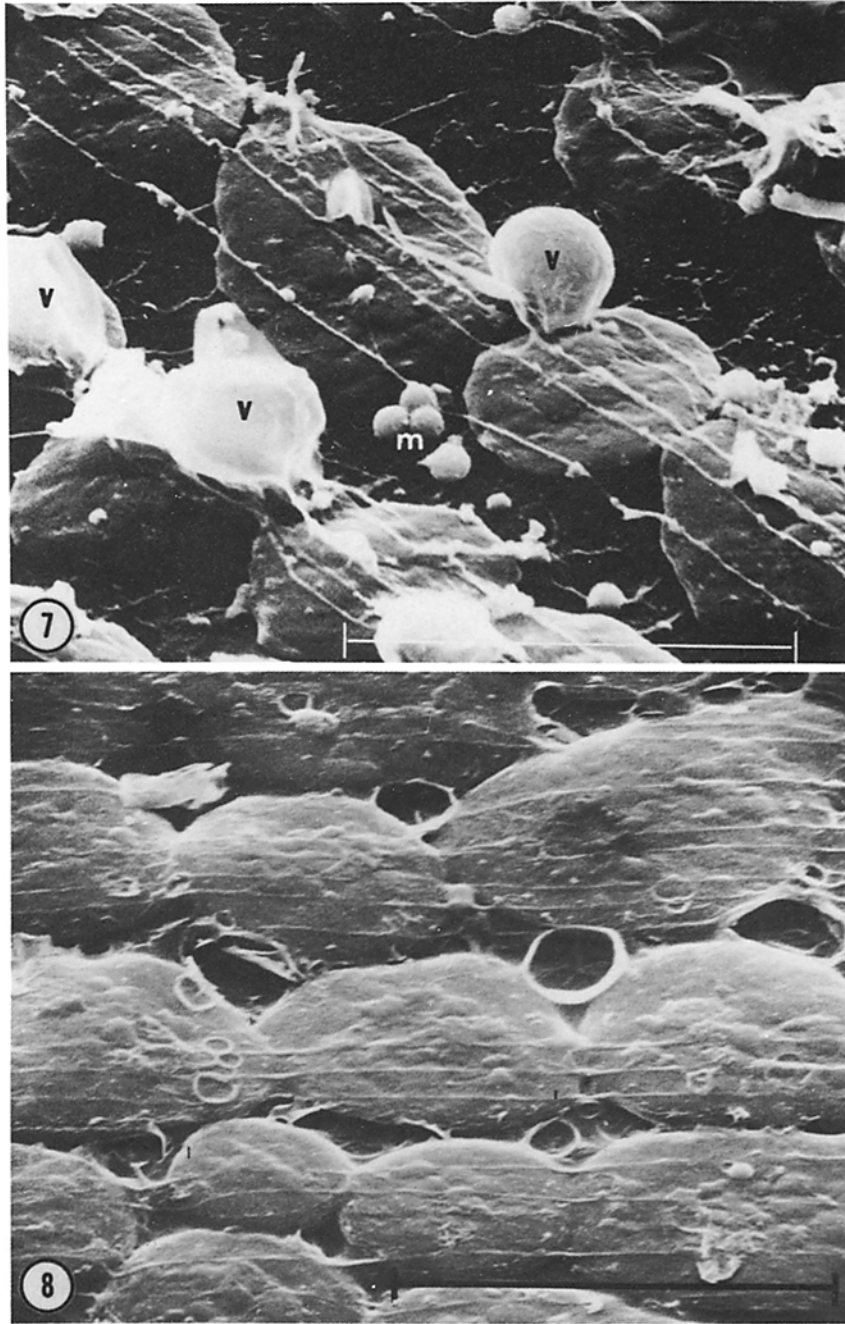
FIGURE 7   SEM of freeze-dried cortex of *C. australis*. Large vesicles of indeterminate nature are present
(v), as well as small spherical organelles some of which represent mitochondria (m), and membrane frag-
ments. Scale = 10 μm, × 6,000.

FIGURE 8   SEM of section of freeze-dried cortex covered by membrane. Fibrils remain taut, which re-
sembles their condition in vivo. Some of the holes in the membrane may be preparation artifacts. Scale =
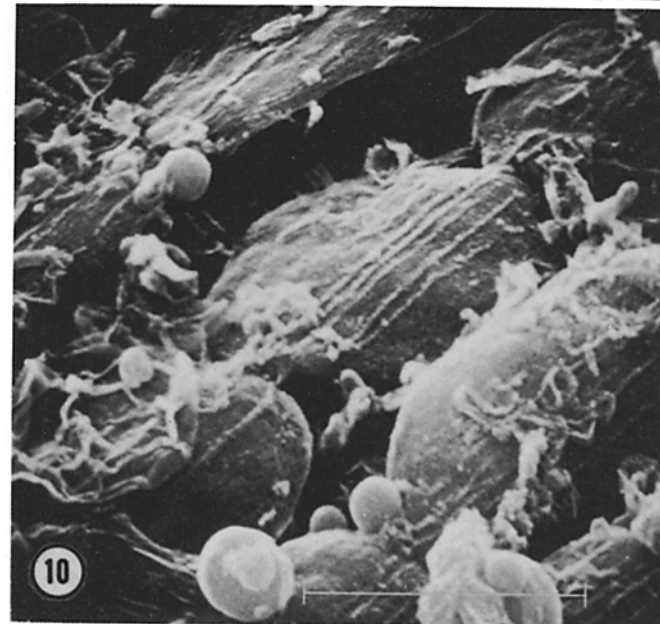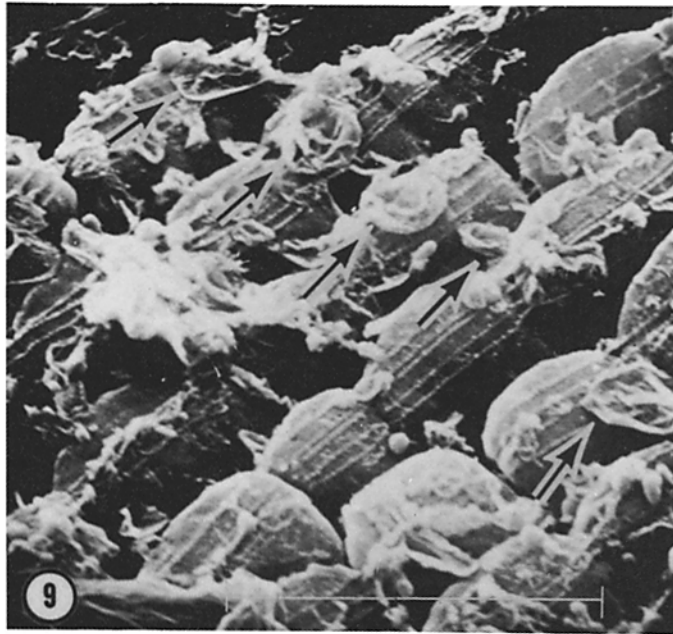10 μm, × 6,000.

FIGURE 9   Cortex of *C. australis* prepared by critical point drying technique. A transverse row of vesicle "ghosts" is evident (arrows). Scale = 10 μm; × 5,000.

FIGURE 10   Higher magnification of critical point-dried material. With this technique, fibrils are usually broken between chloroplasts. Fibrils present a beaded or wavy appearance which may be due to shrinkage during dehydration. Vesicles of indeterminate nature and vesicle "ghosts" are present. Scale = 5 μm; × 7,500.

pattern as is seen in high-resolution light micrographs (18). In vivo, the actin fibrils may be elastic, since chloroplast files have been observed to snap apart under stress conditions (12).

In critical point-dried tissue (Figs. 5, 9, and 10), actin fibrils were usually not visible on the chloroplast surface except in close proximity to masses of cytoplasm covered by membrane, in which loca-

tion they may have been protected from mechanical agitation during CO_2 exchange. Occasionally, however, parallel sets of fibrils can be seen on patches of chloroplasts, but they usually do not extend continuously from one chloroplast to the next in a file. (It may be that where fibrils are preserved, the tonoplast and underlying tissue were sloughed off late in the CO_2 exchange process and served to partially protect the ectoplasmic layer.)

In both freeze-dried and critical point-dried tissue, small spherical or ellipsoidal organelles are visible associated with the chloroplasts and adjacent to the actin fibrils (Fig. 4–10). As discussed previously with respect to negatively stained material, these structures may be either mitochondria, spherosomes, or membrane-bound protein vesicles.

Larger vesicles and "ghosts" are also present in both types of SEM preparations (Figs. 6 *a*, 7, 9, and 10). It is not known what these vesicles correspond to in the living cell. The fate of nuclei is not known; some of the "ghost" vesicles may represent collapsed nuclei.

In both freeze-dried and critical point-dried preparations, actin fibrils are scarce and lack orientation parallel to the one or two rows of chloroplasts adjacent to the clear "indifferent zone" separating the apically and basipetally directed cytoplasmic streams. Also, in both preparation types, an occasional fibril may appear much thinner than the average. Rarely does more than one thin fibril occur per chloroplast file in areas along the lateral walls of these cells.

As discussed previously, even at high magnifications of the SEM, the chloroplast surface shows no specializations suggesting points of actin fibril contact. This is not surprising, since even if surface structures were present, the heavy metal coating could obscure minute details, and, in addition, the resolution of the instrument (ca. 6 nm) would limit visualization of such structures.

DISCUSSION

This research has shown that subcortical actin fibrils in Characean internodes are attached to chloroplasts and occur along the surface tangent to the chloroplast membrane at the ectoplasm-endoplasm interface. We have shown that an average of four to six fibrils are continuous over many chloroplasts in a file, with some extending for long distances along the streaming circuit.

In our experiments, freeze drying appeared superior to the generally preferred critical point-

drying technique in preserving the actin fibrils in these cells.

Each of the techniques employed in this study is subject to artifacts, hence the results require careful interpretation. In the negative staining technique, for example, chloroplasts and fibrils may not adhere tightly to the grid surface and may be removed during rinsing and staining. However, in areas where chloroplasts are well aligned, the fibril number and placement correspond closely to those viewed in SEM preparations and thin sections, so that it seems likely that few fibrils are lost. The negative staining technique is performed in a very few minutes and the actin filaments are organized into bundles; thus, one would not expect fibrils to be lost by actin disaggregation. Since the number of actin filament bundles per chloroplast file is essentially the same in negatively stained preparations as the number identified by SEM as consisting of continuous fibrils, definitive evidence of fibril branching is lacking.

In SEM preparations, endoplasm must be removed to expose the naked actin fibrils, so that endoplasmic branches such as those described by Allen (1) could presumably be peeled off with the endoplasm. However, the generally regular arrangement and relatively uniform diameter of the remaining fibrils do not suggest disruption of this sort. Moreover, in areas where actin fibrils might be expected to be trapped under enveloping membrane (Fig. 8), no branches are discernible.

Thus, even though the study of wave and particle motion along endoplasmic filaments is important to an understanding of the phenomena associated with the force-generating mechanism, it seems probable that fibril undulations as described by Allen (1) are not per se the basic force-generating mechanism in plants. There is considerable evidence that cytoplasmic streaming and particle movement can occur along the subcortical fibrils without visible fibril undulations (for example, references 18 and 35, and our personal observations). Moreover, subcortical fibrils normally attached to the chloroplasts may comprise many, if not most, of the endoplasmic filaments observed in "windowed" cells, since under these conditions actin filaments may enter the endoplasm because they lack an anchor in the ectoplasm. Additionally, endoplasmic branches of the fibrils could be formed by disruption during the "windowing" process. Whatever the origin of endoplasmic filaments, subcortical fibril branches do not appear to be necessary to the streaming mechanism.

Among the alternative mechanisms proposed as

exerting force for cytoplasmic movement in non-muscle cells are propagated changes in packing of actin filaments (16) and actin polymerization-depolymerization cycles (32). However, several factors point to an actin-myosin interaction as being responsible for cytoplasmic movement in plant cells. For example, studies on the behavior of cytoplasmic particles in cell models under various conditions (35) are strongly suggestive of myosin involvement. Also, if we assume that myosin is present, the actin filaments in Characean inter-nodes are favorably oriented for movement of presumptive myosin in the direction of cytoplasmic motion, by analogy to the movement of muscle actin relative to muscle myosin. Thus, the sugges-tion that myosin or a myosin-like enzyme is involved in cytoplasmic streaming in plants (6, 21, 22, 29, 35) seems to be the most promising at this time.

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