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Observations on dividing plastids in the protonema of the moss *Funaria hygrometrica* Sibth.

Arrangement of microtubules and filaments*

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Abstract. The process of division was investigated in the different types of plastids found in the tip cell of the protonema of *Funaria hygrometrica* Sibth. There were no structural changes in the envelope membranes of any of the plastid types during the initial stage of division. As the process of constriction advanced, thylakoids were locally disintegrated and sometimes starch grains in the isthmus were locally dissolved. In the isthmus, tightly constricted plastids were characterized by an undulating envelope and an increasing number of vesicles. After three-dimensional reconstruction of electronmicrographs a distinct filamentous structure was observed in the plane of division outside the plastid but close to the envelope. At different stages of division the constricted regions were partly surrounded by one or a few filaments. The roundish plastids in the apical zone were accompanied by single microtubule bundles, and the spindle-shaped plastids in the cell base were surrounded by single microtubules and microtubule bundles. A model of co-operation between microtubules and the filamentous structure in the division process is discussed.

Key words: Filament (moss) – *Funaria* – Microtubule – Moss (plastid division) – Plastid division – Tip cell (moss).

Introduction

The concept of "plastid continuity" proposed by Schimper (1883) has been verified electronmicroscopically. Plastids increase in number by division or by tying-off, and the course of constriction and fission has been observed in different subjects using the light microscope (Fasse-Franzisket 1955; Green 1964).

Biochemical and genetical analyses indicate that the plastids are semi-autonomous. As a result of the symbiont theory an inner division mechanism has been discussed by Possingham and Lawrence (1983) and such a mechanism is indicated from observations of the inside of dividing plastids (Bell 1982). Experiments with isolated plastids do not prove such an inner mechanism, since constrictions only occur sometimes (Ridley and Leech 1970; Kameya and Takahashi 1971). On the other hand, fuzzy plaques of electron-opaque material (Leech et al. 1981) and ringlike structures of electron-dense material at the envelope membranes of tightly constricted plastids (Chaly and Possingham 1981; Hashimoto 1986) may indicate a co-operation of plastidic and cytoplasmic factors. Recently, Mita et al. (1986), however, described a possible contractile-ring structure outside the chloroplast of the monoplastidic alga *Cyanidium caldarium*. This was the first distinct indication of an outer division mechanism and the involvement of cytoplasmic elements in plastid division. This observation is consistent with the hypothesis that the cytoskeleton takes part in the division process (Brown and Lemmon 1984, 1985).

The protonema of *Funaria hygrometrica* is a convenient object for observing plastid division. Schmiedel and Schnepf (1980) described the polarity of the tip-growing cells which develop from a chloronema to a caulonema growth type. The main reasons for using the different tip cells were the existence of different types of plastids in one cell, and the high frequencies of cell and plastid division. We have tried to establish, by three-dimensional reconstruction of electronmicrographs, a di-

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vision process which is common to the different plastid types, and thereby gain more insight into the mechanism of plastid division.

Material and methods

Plant material and culture conditions. Spore material was a gift of Professor Bopp (Botanisches Institut, Heidelberg, FRG). The protonemata were grown according to the method of Bopp et al. (1964).

Electron microscopy. For electron microscopy, 7- and 14-d-old protonemata were fixed with glutaraldehyde (GA, 2%; Ferak, Berlin, FRG) in Na/K-phosphate-buffer (0.1 M, pH 6.8) for 50 min. In addition to this, 14-d-old protonemata were fixed with GA and tannic acid (0.5%; EMS, Fort Washington, Pa., USA). Osmium tetroxide (OsO_4, 2%; Ferak) was used for 2 h postfixation. Tip cells, embedded in Spurr's resin, were selected by light microscopy, excised and remounted for further examination by electron microscopy. Longitudinal and transverse serial sections of whole tip cells were examined with a Siemens (Berlin, West Germany) ELMISKOP 101. Dividing plastids and the surrounding cytoplasm were reconstructed three-dimensionally to obtain information about the arrangement of the cytoskeleton. Sections were drawn on transparent film and glass panes were placed between the drawings of successive sections. Stereographs were recorded.

Inhibitor experiments. Protonemata were excised and transferred on cellophane film into Petri dishes which contained culture medium with 0.1 $\mu g \cdot ml^{-1}$ colchicine (Sigma, Taufkirchen, FRG; Schmiedel and Schnepf 1980). In the same way, cells were exposed to cytochalasin B (Aldrich, Steinheim, FRG) at 20 $\mu g \cdot ml^{-1}$ concentration, dissolved in 1% DMSO (dimethylsulfoxide; Serva, Heidelberg, FRG; Schmiedel and Schnepf 1979). Tip cells were observed by Nomarski interference contrast microscopy with a Zeiss Photomikroskop III (Zeiss, Oberkochen, FRG).

Results

The polarly organized tip cells of *Funaria* consist of apical and basal zones characterized mainly by plastids, and by vacuoles and plastids, respectively (Fig. 1; Schmiedel and Schnepf 1980). The nucleus is positioned between these zones. Chloronema tip cells (7-d-old protonema) contain roundish chloroplasts in the apical zone and spindle-shaped ones in the basal zone (Fig. 1a). Both types of chloroplast contain a thylakoid system which is extended parallel to the long axis of the organelle (Figs. 2, 3a).

Three types of plastid occur in the caulonema tip cells (14-d-old protonema, Fig. 1b). These plastids are characterized by low numbers of thylakoids. A group of approximately ten spherical amylochloroplasts in the apical region tends to lie on the bottom of the cell in horizontally grown protonemata (Schmiedel and Schnepf 1979). These spherical plastids contain large starch grains and few inner membranes (Fig. 4a). The majority of the plastids in the apical zone are randomly distributed roundish plastids. The basal zone contains spindle-shaped plastids with few starch grains.

In all the different groups of plastids there were always some in the state of division, those in the apical zone showing the highest rate of division.

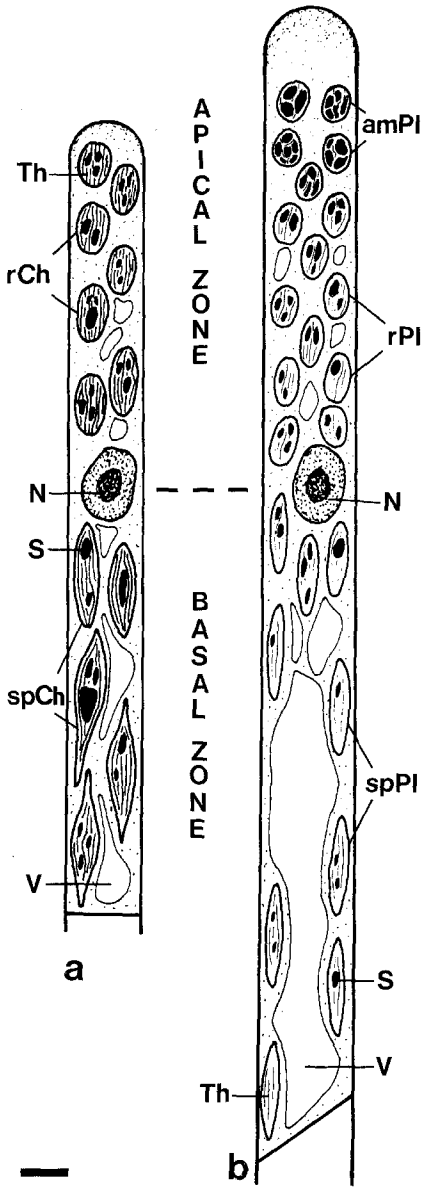


Fig. 1a, b. Schematic drawing of a chloronema and a caulonema tip cell. The distribution and structure of the different plastid types are illustrated (the dimensions of the plastids are not to scale). No division states are depicted. **a.** The apical zone of the chloronema tip cell contains roundish chloroplasts (*rCh*), the basal zone spindle-shaped chloroplasts (*spCh*). **b.** The apical zone of the caulonema tip cell possesses two types of plastids, amylochloroplasts (*amPl*) and roundish plastids (*rPl*). The basal zone contains spindle-shaped plastids with few thylakoids (*spPl*). *Th*, thylakoid; *S*, starch grain; *N*, nucleus; *V*, vacuole. Bar = 10 μm

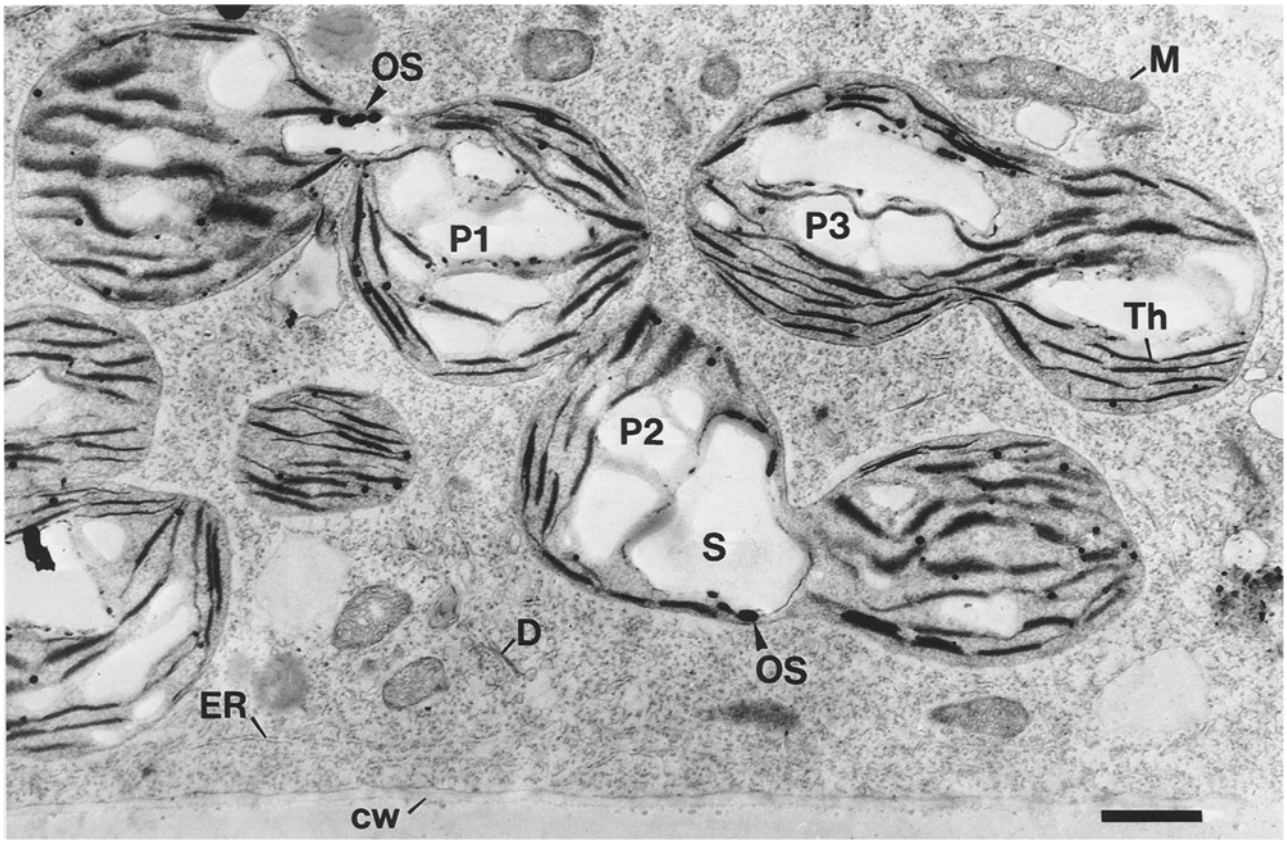
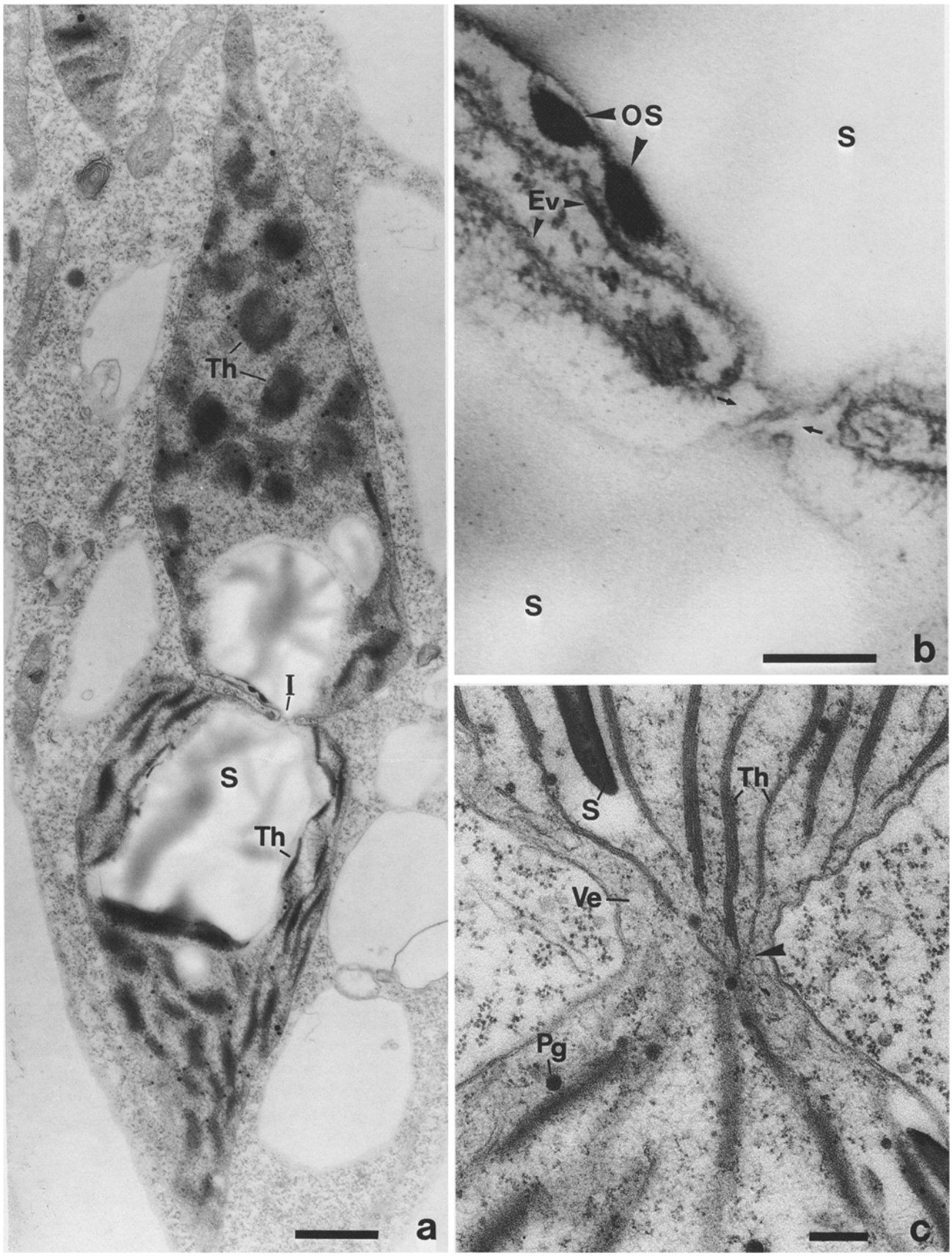


Fig. 2. Apical part of a *Funaria* chloronema tip cell. Three dumbbell-shaped, polarly organized plastids (*P1, P2, P3*) are oriented parallel to the cell wall (*CW*). The chloroplasts contain large starch grains (*S*) which partly disturb the parallel arrangement of the thylakoids (*Th*). *P3* represents an early division state, *P1* is tightly constricted. Mainly in the isthmus region (*P1, P2*), osmiophilic structures (*OS*) are positioned near the surface of starch grains. *M*, mitochondrion; *D*, dictyosome; *ER*, endoplasmic reticulum. Bar = 1 μm; × 13000

Neither a division rhythm nor a temporal interrelation between plastid and cell division was observed (compare Jensen 1981; Jensen and Jensen 1984). The dividing plastids of the chloronema and the caulonema show no distinct structural differences, all types dividing in a similar way. A constriction divides the organelle into two daughter halves of similar size. Frequently, the two halves include an unequal amount of starch (Fig. 2), as can be proved by serial sections.

In the early stages of division there is a smooth depression of the plastid envelope and a constriction is then formed at right angles to the longitudinal axis of the organelle. The inner structure of these chloroplasts is unchanged and the envelope membranes do not display any obvious structural differences compared with non-dividing plastids. Dumbbell-shaped plastids show disintegrated thylakoids in the isthmus near the envelope (Fig. 3c). In the region of constriction, starch grains are sometimes deeply incised (Fig. 3a). Osmiophilic structures are located on the surface of the starch grains in the isthmus (Fig. 2, 3b). The envelopes of tightly constricted plastids show invaginations in the region of the isthmus. Numerous vesicles were observed in the narrow isthmus (Fig. 5; compare also Suzuki and Ueda 1975). There are some additional characteristics and exceptions to the above description that should be mentioned. The longitudinal axis of some tightly constricted plastids is bent at the isthmus, which is decentrally positioned in many plastids in the chloronema and the caulonema apical zone (Fig. 2). Frequently, the longitudinal axis of the constricted amylochloroplasts is not parallel to the cell axis. Single sections and three-dimensional reconstructions prove that, occasionally, the orientation of the thylakoids in the two halves of severely constricted chloroplasts is different (Fig. 3a).

Filamentous structures. The isthmus of dividing plastids was partly surrounded by filamentous structures in the plane of division (Fig. 4a–c, 6a–f). Similar structures could be observed in all de-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scribed areas of chloronema and caulonema tip cells. The cross-sectioned filamentous structures were 10–40 nm in width (Fig. 7a) and 10–15 nm thick (Fig. 7b), and ran parallel to the outer envelope at a distance of about 10 nm. The preserved structure ended where the distance between the structure and the envelope increased.

The three-dimensional reconstruction of the constricted plastids shows the arrangement of the filamentous structures (Fig. 8a–f). Frequently, a semicircular structure was observed (8a). Sometimes a split end occurred (8b). Further arrangements included two parallel semicircles (8c) or two structures with a crossing point (8d). Some plastids were surrounded by two independent structures on opposite sides of the constricted region (8e). Helical structures were reconstructed (8f), too. Often, the ends of the structures were directed into the cytoplasm, but no closed plastid-dividing ring structure, as recently reported by Mita et al. (1986), was ever reconstructed. Frequently, a single filamentous structure was observed on the tigthly constricted side of a decentrally positioned constriction region, whereas a helical structure or two independent semicircular structures often surrounded a centrally positioned isthmus. Cross-bridge-like structures seemed to exist between the outer envelope and the filamentous structures (Fig. 9).

Cytochalasin-B treatment. The inhibitor cytochalasin B affected caulonema tip cells earlier and to a greater extent than chloronema tip cells. The growth rate was reduced but the distribution and the shape of the plastids remained unchanged. Only a few plastids were observed in the state of division.

Microtubule system. The addition of tannic acid to the fixation solution resulted in good preservation of microtubules. The following details are based on observations on caulonema tip cells. The cells contain two populations of microtubules: cortical microtubules are located close to the plasmalemma and parallel to the cell axis; non-cortical microtubules are distributed in the central cytoplasm and preferentially run parallel to the cell axis. A great number of non-cortical microtubules was observed in the vicinity of the nucleus (Schmiedel et al. 1981).

Only a few single non-cortical microtubules were observed between the amylochloroplasts. A few bundles of microtubules and single microtubules partly accompany the randomly distributed plastids in the apical zone. The basal part of the cell contains a distinct microtubule system with bundles of three to five microtubules running parallel to the spindle-shaped plastids (Fig. 9, 10). Often, we observed plastid poles framed by single microtubules (Fig. 11a). The plastids and the microtubules are connected by cross-bridging structures (Fig. 11a, b), whose length ranges from 15 to 40 nm. Microtubules and filamentous structures run nearly perpendicular to each other, and sometimes single microtubules are located close to the filamentous structures (Fig. 12). No direct structural connection between microtubules and the filamentous structure has been observed.

Colchicine treatment. A few minutes after transferring the protonemata onto the colchicine-containing medium, the growth and polarity of the tip cells were disturbed (Schmiedel and Schnepf 1979). Similar to the cytochalasin-B effect, colchicine affected the caulonema tip cell earlier and to a greater extent than the chloronema tip cell. After 3 h, plastids accumulated in the club-shaped tip. The basal zone of the cell was also characterized by groups of accumulated plastids. The spindle-shaped plastids became rounded-off and some plastids showed an amoeboid shape. A few constricted plastids were recognized. Five hours later, however, growth was strongly inhibited and no division stages could be observed.

Discussion

Schmiedel and Schnepf (1980) pointed out the interrelation between cell polarity and organelle distribution in *Funaria* cell threads. They described the different plastid types as an attribute of the polarly organized caulonema tip cell. The results

Fig. 3. a Median section of a serially sectioned, tightly constricted, spindle-shaped chloroplast in the basal zone of a *Funaria* chloronema tip cell. The disk-like grana-thylakoids (*Th*) in the upper half are arranged parallel to the section plane. The thylakoids in the lower half are more perpendicular to the section plane. A central starch grain (*S*) is deeply intersected. The isthmus (*I*) has a diameter of 230 nm. Bar = 1 μm; × 14000. **b** Detail of the constriction region. Exceptionally, the envelope (*Ev*) shows no invaginations. The starch grain (*S*) is not completely divided, and it seems to have been ruptured (→) during preparation, the shape of the grain indicating the process of local dissolution. Osmiophilic structures (*OS*) are positioned on the surface of the grain. Bar = 0.2 μm; × 100000. **c** Median section of a constricted chloroplast in the apical zone of a chloronema tip cell. Thylakoids are locally disintegrated (►). *S*, starch grains; *Ve*, vesicles in the isthmus; *Pg*, plastoglobuli. Bar = 0.2 μm; × 50000

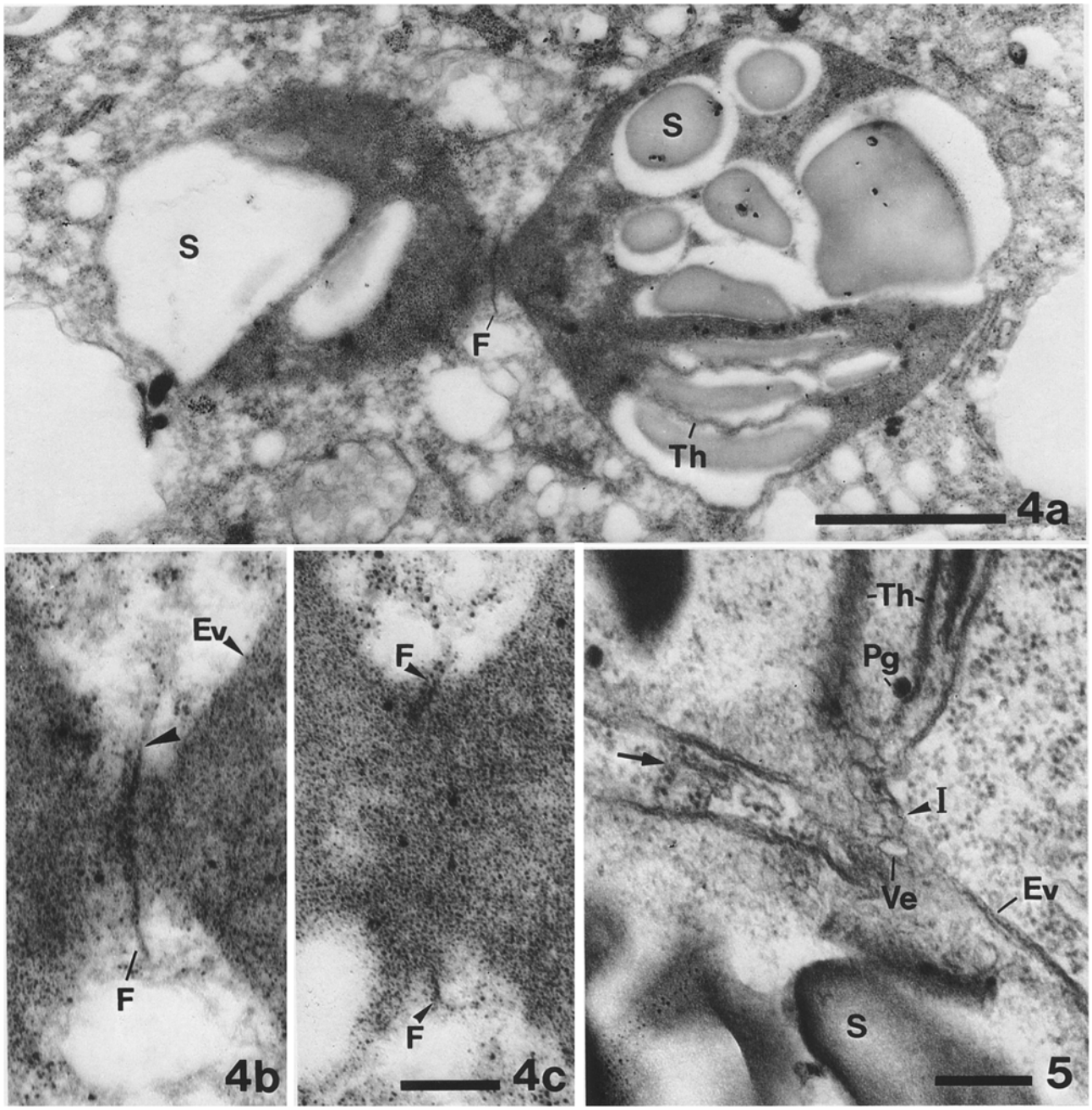
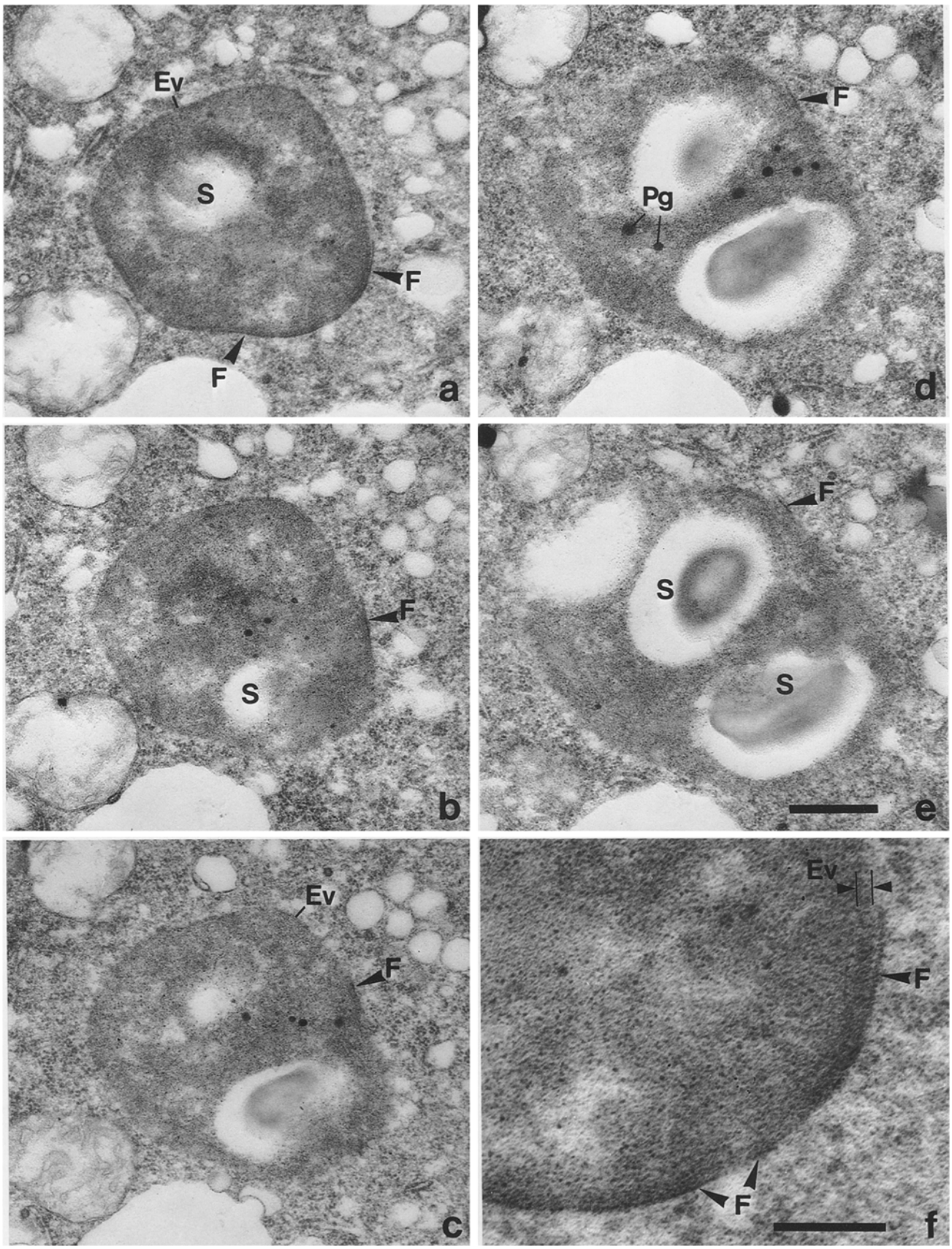


Fig. 4. **a** Longitudinal section of a dividing amylochloroplast in a *Funaria* caulonema tip cell. The left half and the isthmus of the plastid are sectioned at the periphery, whereas the right half is centrally cut. The plastid contains many starch grains (*S*), but thylakoids (*Th*) are hardly visible. In the plane of division, the isthmus is partly surrounded by a filamentous structure (*F*). Bar = 1 μm; × 29000. **b** Detail of the isthmus region. The single filamentous structure has a width of 15 nm, but at the split end the width is 35 nm (►), each branch having a width of 15 nm. *Ev*, envelope. **c** Continuing parts (*F*) of the filamentous structure: *at the bottom*, a single strand; *on top* two parallel strands. Bar = 0.2 μm; × 78000

Fig. 5. Detail of a tightly constricted chloroplast in the apical zone of a *Funaria* chloronema tip cell. The median section shows the isthmus (*I*) with a diameter of 190 nm. In the region of constriction, the envelope (*Ev*) is more or less undulating. The isthmus contains vesicles (*Ve*). Outside the chloroplast in the vicinity of the isthmus, membranous material is located (→). *Th*, thylakoids; *S*, starch grain; *Pg*, plastoglobulus. Bar = 0.2 μm; × 74000

Fig. 6a–f. A series of five transverse sections from the isthmus of a constricted plastid in the apical zone of a *Funaria* caulonema tip cell. The transverse sections of the isthmus are partly surrounded by a filamentous structure (*F*) close to the envelope (*Ev*). Sometimes, the addition of tannic-acid to the fixation solution results in a bad preservation of membranes. The longest part of the filamentous structure is shown in **a**, four continuing parts are visible in **b–e**, and the end **e** projects into the cytoplasm. The isthmus contains starch grains (*S*) and plastoglobuli (*Pg*). Bar = 0.5 μm; × 32000. **f** Detail from **a**. Outside the isthmus, a distinct filamentous structure (*F*) is arranged close to the envelope (*Ev*). Bar = 0.2 μm; × 100000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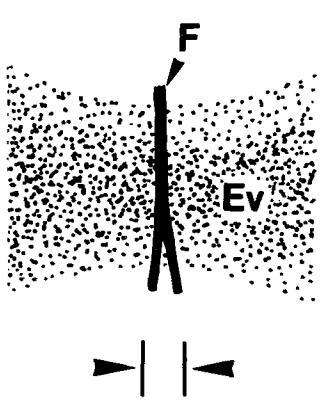

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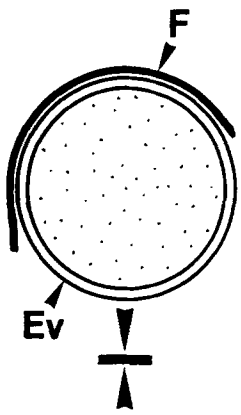

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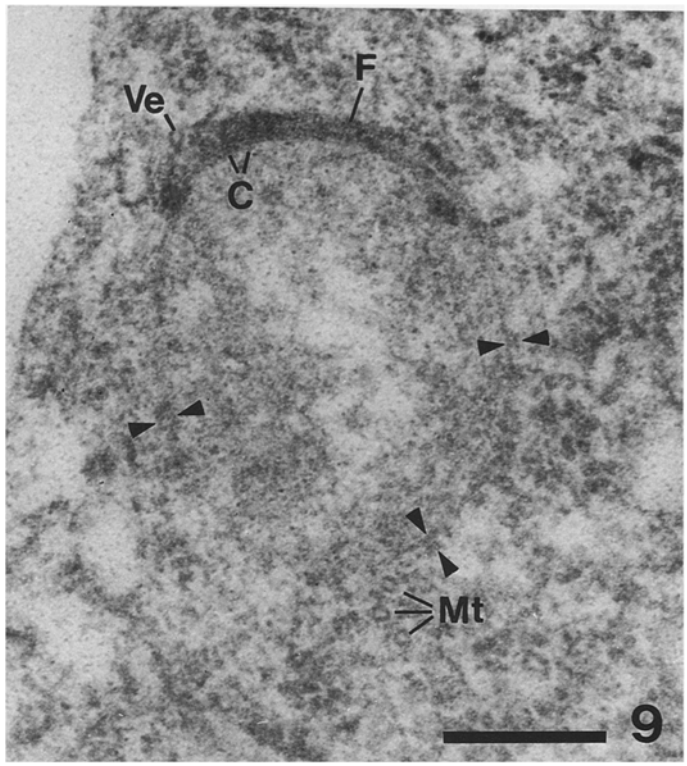

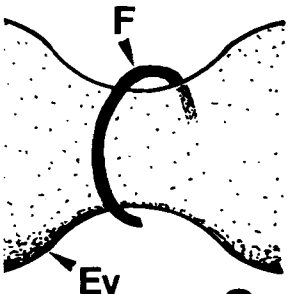

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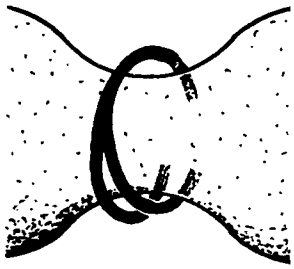

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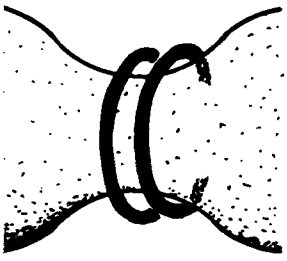

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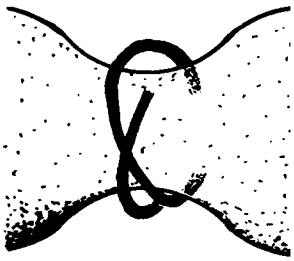

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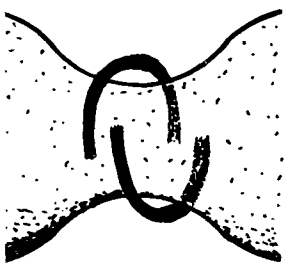

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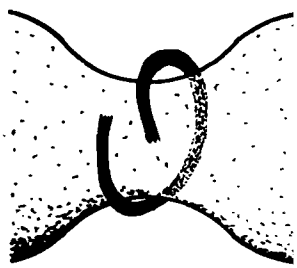

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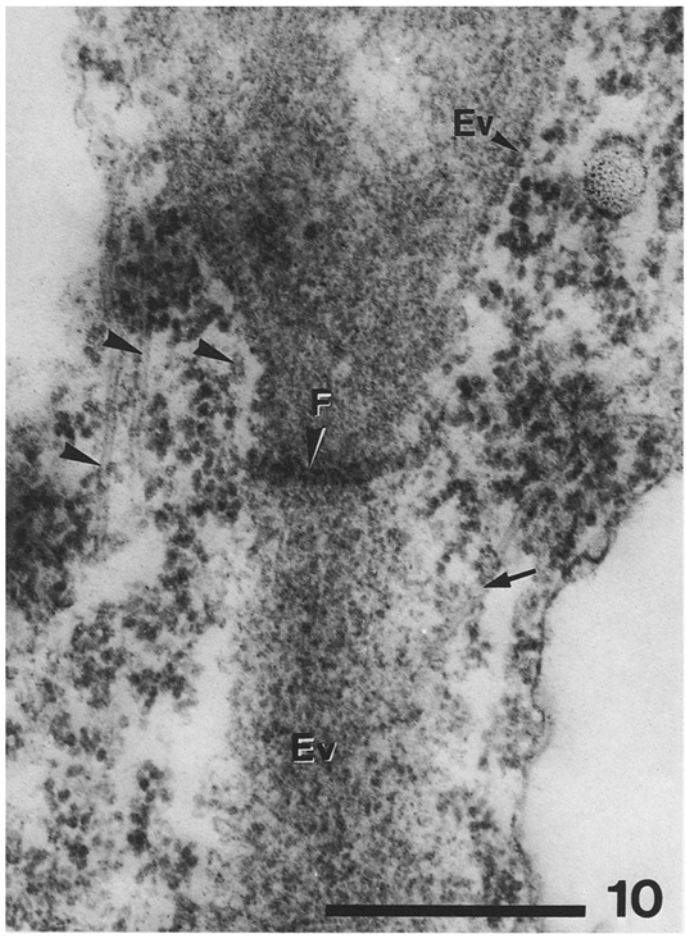

of the present investigations have shown that, regardless of differences in position, shape and inner structure, all the described types of chloronema and caulonema plastid are able to divide, and that during division the succession of events is similar.

Three possible plastid-division mechanisms are worthy of consideration. These are an outer cytoplasmic and an inner plastidic mechanism and, finally, a mechanism whereby cytoplasmic and plastidic factors may co-operate. The first observable event, the smooth constriction of the plastid, may indicate the action of an outer force. In relation to this, the cytoskeleton may be an important factor in plastid division, since the described filamentous structures have only been observed at dividing plastids and indicate a cytoplasmic division mechanism. A simple mechanism would involve a contractile-ring structure such as that described by Mita et al. (1986) at the dividing chloroplast of the monoplastidic alga *Cyanidium caldarium*. They supposed that the increasing thickness of the structure during the division process resulted from a contraction during the advancing constriction. We did not observe a ring structure. The different arrangements of the filamentous structure, especially the semicircular ones, indicate an anchoring in the cytoplasm to produce a constricting force. A connection with non-cortical or cortical microtubules or other cytoplasmic elements could not be observed, because only filamentous parts close to the envelope were preserved. The results indicate that fixation with glutaraldehyde and tannic acid preserves only parts of the filamentous system, probably consisting of tiny filaments. Our results show no relation to the electron-dense deposits observed by Leech et al. (1981), Chaly and Possingham (1981) and Hashimoto (1986).

Glutaraldehyde destabilizes microfilaments and the addition of tannic-acid to the fixation solution causes an increasing number of observable cytoskeletal elements (Seagull and Heath 1979). The observed filamentous structures run near the envelope and end when their distance from the envelope increases. A stabilizing contact or their arrangement close to the envelope may be responsible for the preservation of these filamentous structures. The simultaneous good staining of both the microtubules and the filamentous structures may indicate the microfilament character of the latter. A single filament seems to be 10–15 nm thick, and two or more parallel filaments may cause the range of widths (15–40 nm), as indicated by the split structures (Fig. 4b). Contraction of the filamentous structure itself should result in an increasing thickness during the advancing constriction, but this was not observed.

A one-sided pulling force could be the reason for the deeper incision on the side surrounded by a semicircular structure, and could also cause decentrally positioned isthmuses and the bent longitudinal axis of some tigthly constricted plastids. Filamentous structures which completely surround an isthmus would cause a symmetrical traction.

Treatment with cytochalasin B reduces growth and, consequently, a high rate of plastid division is no longer required to guarantee a distinct organelle distribution. There was no clear indication of whether or not actin is necessary for plastid constriction, although preliminary immunofluorescence experiments indicated the presence of an actin system in *Funaria* tip cells (data not shown). The involvement of an actin system in plastid division has still to be investigated. Nevertheless, the shape of the isthmuses and the different configura-

Fig. 7a, b. Schematic drawing to show the dimensions of the filamentous structures. **a** Longitudinal section of a division stage. In peripheral sections of the isthmus, the widths of the filamentous structures (*F*) range from 10 to 40 nm. **b** In transverse sections of the isthmus, the filamentous structures measure 10–15 nm in thickness and are arranged parallel to the envelope (*Ev*)

Fig. 8a–f. Arrangement of the filamentous structures reconstructed from serial sections (schematic drawing). The isthmus is surrounded by filamentous structures (*F*) close to the envelope (*Ev*). **a** A semicircle. **b** A semicircle with a split end. **c** Two parallel semicircles. **d** Two structures with a crossing point. **e** Two semicircles surround the isthmus on opposite sides without contact. **f** A helical structure surrounds the whole isthmus

Fig. 9. Transverse section in the region of constriction of a spindle-shaped plastid in the basal zone of a *Funaria* caulonema tip cell. A bundle of microtubules (*Mt*) is cross-sectioned opposite to the filamentous structure (*F*). The envelope (►◄) is hardly visible (compare **Fig. 6**). Cross-bridging structures (*C*) are indicated between the filamentous structure and the envelope. *Ve*, vesicle, proved by the section series. Bar = 0.2 μm; × 106000

Fig. 10. Longitudinal section of a constricted spindle-shaped plastid in the base of a *Funaria* caulonema tip cell. The elongated isthmus is sectioned at its periphery and the envelope (*Ev*) is partly positioned in the section plane. Parallel to the longitudinal axis, a microtubule bundle (►) accompanies the isthmus. *Right:* a single tubule (→). A distinct filamentous structure (*F*, width 35 nm) surrounds the narrowest part of the constriction region. Bar = 0.5 μm; × 68000

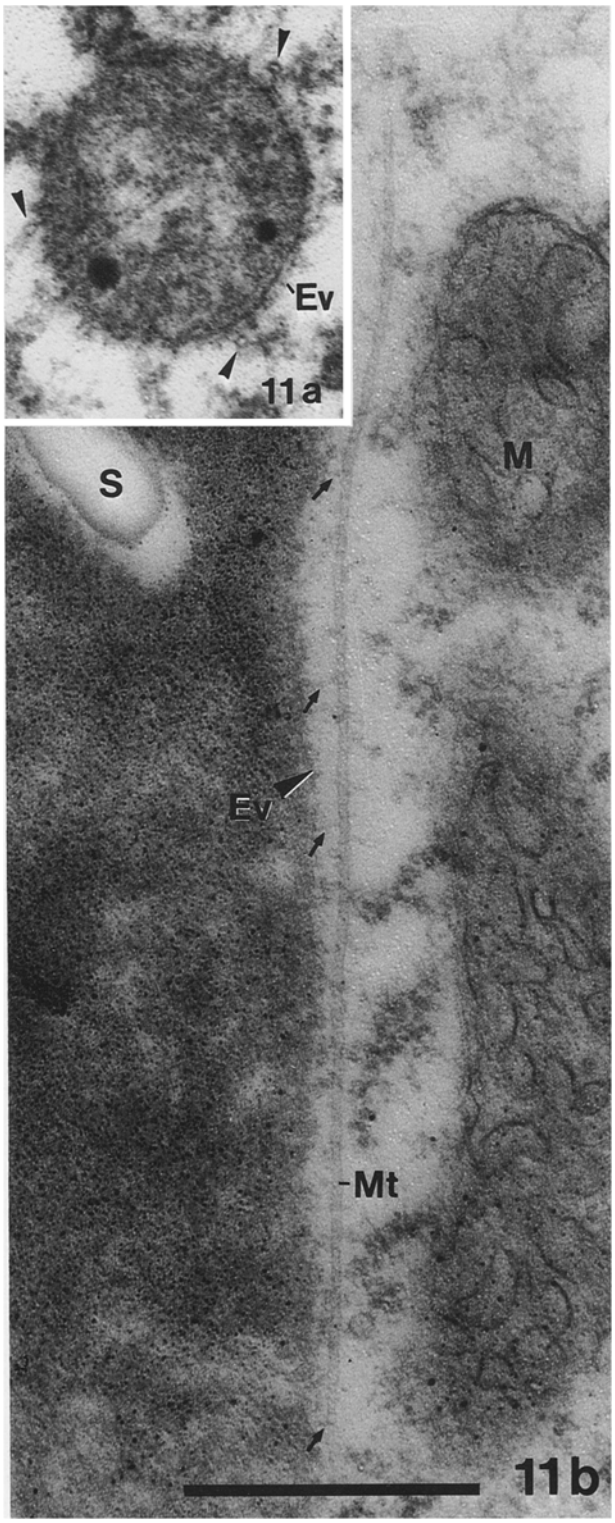

Fig. 11a, b. Details of plastids at the base of a *Funaria* caulonema tip cell. **a** Cross-sectioned plastid pole framed by three single microtubules (►) which are connected to the envelope (*Ev*) by cross-bridging structures. **b** The left side shows part of a longitudinally sectioned plastid. The envelope (*Ev*) is hardly visible (compare **Fig. 6**). A 1.8-μm-long part of a microtubule (*Mt*) is arranged parallel to the plastid. Cross-bridging structures (→), up to 40 nm long, connect the microtubule and the envelope. *M*, mitochondrion; *S*, starch grain. Bar = 0.5 μm; × 79000

tions of the filamentous structure support the assumption that it participates in the process of plastid division.

The plastids may be anchored to the microtubule tracks by the observed protein cross-bridges which are similar to those described between microtubules and the endoplasmic reticulum (Franke 1971) and between microtubules and the plasmalemma (Hensel 1984). Our results support the conclusion that the microtubule system in *Funaria* participates in nuclear movement and in the migration and anchoring of chloroplasts (Schmiedel and Schnepf 1980; Schnepf et al. 1982). The direct participation of microtubules in the process of constriction can be excluded because the microtubules are mainly oriented parallel to the plastid axis. Furthermore, only a few single microtubules were observed in the vicinity of the constricted amylochloroplasts. The microtubule system may, however, draw apart the daughter halves of the spindle-shaped plastids. This process would cause elongated constriction regions. Such constriction regions were observed with spindle-shaped chloroplasts in the basal zone of tip cells (Fig. 12). Colchicine treatment indicates that microtubules influence the shape of the plastids. The remaining polar arrangement of the plastids after cytochalasin-B treatment supports our conclusions. Colchicine treatment does not help to clarify the mechanism of plastid division.

Our knowledge of the interplay between microfilaments and microtubules is rather fragmentary. Hepler and Palevitz (1974) suggested that the microtubule and microfilament systems functioned antagonistically, and inhibitor experiments support this assumption (Schmiedel and Schnepf 1980). This is in contradiction to the results of Menzel and Schliwa (1986a, 1986b). The green alga *Bryopsis* possesses a highly organized cytoskeleton within which the co-operation of microfilaments and microtubules affects the migration of the plastids; microtubule-associated proteins may be the interactors (Griffith and Pollard 1978). Our results do not prove structural connections between the two different cytoskeletal elements. However, we suggest plastid division in *Funaria* is based on the co-operation of different cytoskeletal elements. The filamentous structure may be connected to microtubular tracks in the cell base and to non-cortical or cortical microtubules in the

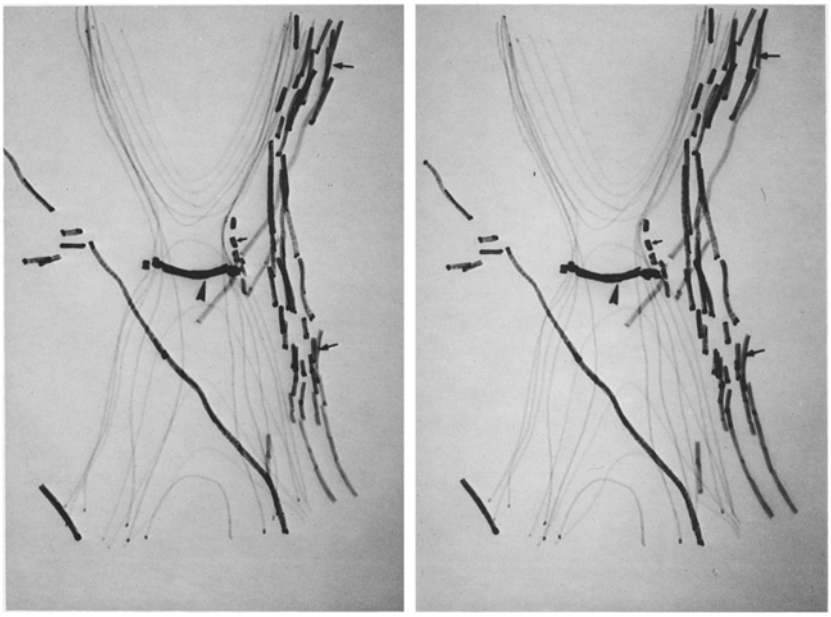

Fig. 12. Three-dimensional reconstruction of an isthmus and the cytoskeletal elements (stereographs, *light lines* show the envelope). The narrowest part of the isthmus is surrounded by a semicircular filamentous structure (*arrowhead*). A microtubule bundle is arranged parallel to the longitudinal axis of the plastid (large arrow). One microtubule is positioned close to the filamentous structure (*small arrow*). Using conventional stereo magnifying glasses, the figure and the arrows will be enlarged 2–3 times

apical zone. Microtubules may be the anchor for the constricting filamentous structures, probably actin.

On the other hand, processes inside the plastids have to be taken into consideration. During the advancing constriction, thylakoids are locally disintegrated from the periphery to the center and starch grains in the isthmus are locally dissolved. Both processes are indicative of a complex enzymatic mechanism in the stroma of the plastids. Enzymes involved in starch dissolution may be located at the osmiophilic structures which accumulate mainly on the surface of the starch grains near the isthmus (Fig. 3b). The eventual state of plastid division seems to be initiated by local instability of the envelope membrane. Invaginations and vesicles were observed inside the isthmus. The fission of the dumbbell-shaped plastids seems to be affected by spontaneous membrane rupture and subsequent fusion. These conclusions correspond to the hypothetical fluid mechanical model of plastid division based on spontaneous and controlled steps as suggested by Possingham and Lawrence (1983).

The essential aspect of this study is the observation of a cytoplasmic structure involved in plastid division, indicating the participation of nuclear controlled structures in the dividing process of semiautonomic organelles. This excludes an independent, inner division mechanism of the plastids. The plastid-dividing structure together with the disintegration processes inside the plastids indicate a complex control mechanism for the division process.

The mechanism triggering the division process remains questionable. The specific local distribution of the plastids possibly causes the initial step of the division. On the other hand, Leech et al. (1981) suggest that changes of envelope membrane tension trigger the division process.

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