Protoplasma (1990) 159: 60–69

PROTOPLASMA

Microtubule distribution in gravitropic protonemata of the moss *Ceratodon*

J. Schwuchow[1], F. D. Sack[2,*], and E. Hartmann[1]

[1] Institut für Allgemeine Botanik, Universität Mainz, Mainz, and [2] Department of Plant Biology, Ohio State University, Columbus, Ohio

Received December 19, 1989
Accepted August 8, 1990

Summary. Tip cells of dark-grown protonemata of the moss *Ceratodon purpureus* are negatively gravitropic (grow upward). They possess a unique longitudinal zonation: (1) a tip group of amylochloroplasts in the apical dome, (2) a plastid-free zone, (3) a zone of significant plastid sedimentation, and (4) a zone of mostly non-sedimenting plastids. Immunofluorescence of vertical cells showed microtubules distributed throughout the cytoplasm in a mostly axial orientation extending through all zones. Optical sectioning revealed a close spatial association between microtubules and plastids. A majority (two thirds) of protonemata gravistimulated for > 20 min had a higher density of microtubules near the lower flank compared to the upper flank in the plastid-free zone. This apparent enrichment of microtubules occurred just proximal to sedimented plastids and near the part of the tip that presumably elongates more to produce curvature. Fewer than 5% of gravistimulated protonemata had an enrichment in microtubules near the upper flank, whereas 14% of vertical protonemata were enriched near one of the side walls. Oryzalin and amiprophos-methyl (APM) disrupted microtubules, gravitropism, and normal tip growth and zonation, but did not prevent plastid sedimentation. We hypothesize that a microtubule redistribution plays a role in gravitropism in this protonema. This appears to be the first report of an effect of gravity on microtubule distribution in plants.

Keywords: Gravity; Microtubules; Protonema; *Ceratodon;* Gravitropism.

Abbreviations: APM amiprophos-methyl; DIC differential interference contrast; DMSO dimethyl sulfoxide; EGTA ethylene glycol-bis-(β-amino-ethylether) N,N,N',N'-tetraacetic acid; FITC fluorescein isothiocyanate; GS gravitropic stimulus; MT microtubule; PIPES piperazine-N,N'-bis-2-ethanesulfonic acid.

Introduction

Tip cells of protonemata of the moss *Ceratodon purpureus* are negatively gravitropic in the dark and positively phototropic under directional illumination (Hartmann et al. 1983). *Ceratodon* protonemata are an attractive system for studying gravitropism because all stages – perception, transduction, and the response (curvature) – occur within the same cell. With the exception of *Chara* rhizoids (Bartnik and Sievers 1988) little is known about gravitropism in tip-growing cells (e.g., Banbury 1962, Schnepf 1986).

Horizontal placement (gravistimulation) results in plastid sedimentation close to the tip in *Ceratodon* protonemata (Walker and Sack 1990). A great deal of evidence suggests that plastids that sediment are important for gravitropic sensing (Volkmann and Sievers 1979, Kiss and Sack 1989, Sack and Kiss 1989), perhaps through interaction with the cytoskeleton (White and Sack 1990). Microtubules are associated with plastids in the tip cells of (non-gravitropic) *Funaria* protonemata (Tewinkel and Volkmann 1987, Wacker et al. 1988), and microtubule inhibitors appear to affect protonemal growth, zonation, and plastid position (Wacker et al. 1988).

Physcomitrella protonemata are gravitropic and have abundant microtubules, but the effect of protonemal reorientation upon microtubule distribution was not reported (Jenkins et al. 1986; Doonan et al. 1985, 1988). Since microtubules seem to be important for oriented (polar) protonemal growth, we used immunofluorescence to study the relationship between microtubules and plastids, and the effect of gravity upon the distribution of microtubules in the gravitropic protonemata of *Ceratodon*. We report here that microtubules are present close to plastids and that horizontal placement results in an apparent enrichment in microtubules near the lower flank of the tip cell.

* Correspondence and reprints: Department of Plant Biology, Ohio State University, 1735 Neil Avenue, Columbus, OH 43210-1293, U.S.A.

Materials and methods

Plant material

Ceratodon protonemata were grown on sterile cellophane for 5–6 days in vertically maintained petri dishes containing a modified Knop's agar medium (Hartmann et al. 1983). Protonemata grew straight up during this period if kept in total darkness. Protonemata were gravistimulated at 90° either by rotating the entire dish (in darkness) or by turning the cellophane (with attached protonemata) 90° in dim light (less than 5 mW/m^2 for 1 min).

Microtubule inhibitors

A stock solution of the microtubule inhibitor oryzalin (1 mM in ethanol; Eli Lilly Co., Indianopolis, Indiana, U.S.A.) was diluted with distilled water to a final concentration of 1 μM oryzalin and 0.1% ethanol (v/v). Controls were treated with 0.1% ethanol in water which corresponded to the concentration of ethanol in the solution of oryzalin with the highest concentration (1 μM) which produced no observable effects. The stock solution of APM (3 mM in DMSO; Mobay Corporation, Kansas City, Missouri, U.S.A.) was diluted with distilled water to a final concentration of 3 μM APM and 0.1% DMSO (v/v). Controls were treated with 0.1% DMSO in water corresponding to the highest final concentration of APM (3 μM) which produced no observable effects. The inhibitors were applied directly onto the protonemata grown on cellophane.

Immunofluorescence

Vertical or gravistimulated protonemata were fixed in place in dim light for 6–10 min with 3.7% (w/v) paraformaldehyde in 0.1 M PIPES buffer, 5 mM EGTA, pH 6.8. In some cases, a phosphate buffer was used (50 mM K_2HPO_4, 50 mM KH_2PO_4, 5 mM EGTA, pH 6.8). The protonemata, which were still attached to the cellophane, were then briefly rinsed in buffer and transferred to polylysine-coated glass coverslips (0.1% aqueous solution of polylysine hydrobromide; Polysciences, Warrington, Pennsylvania, U.S.A.). Care was taken to maintain the original orientation of the cells (either vertical or gravistimulated) during all stages of processing. Protonemal cell walls were then digested with 0.5% (w/v) cellulase Y-C (Seishin Pharmaceutical Co., Tokyo, Japan) and 0.5% (w/v) pectinase (Sigma Chemical, St. Louis, Missouri, U.S.A.) in 0.4 M mannitol, 0.3 mM phenylmethyl-sulfonyl fluoride, and 50 μg/ml leupeptin, in buffer. Protonemata were then rinsed in a buffer consisting of phosphate buffered saline containing 0.1% (w/v) bovine serum albumin. Cells were then incubated in a monoclonal rat anti-yeast tubulin antibody (Sera-Lab/Accurate Chemical, Westbury, N.Y., U.S.A.) diluted 1 : 60 in buffer for 1 h. After three buffer rinses, protonemata were incubated in a goat anti-rat secondary antibody conjugated to FITC (Accurate Chemical) diluted 1 : 60 for 1 h. Protonemata (coverslips) were rinsed in buffer and mounted in Mowiol (Calbiochem, Behring Diagnostics, La Jolla, California, U.S.A.) containing 0.04% (w/v) paraphenylenediamine and the coverslips were sealed to the slides. Tubulin fluorescence was examined at least 12 h later (to allow time for the action of the anti-fading agent), using a 450–490 nm band pass exciter filter, a 510 nm dichroic beam splitter, and a 515 nm long pass barrier filter. Fluorescence was viewed with a Leitz NPL Fluotar NA 1.32 × 100 oil immersion objective using a Zeiss IM35 inverted microscope. Conjugate images were obtained using modified differential interference contrast (DIC) optics. Micrographs were recorded on Kodak Plus X film developed with Diafine (Acufine Inc., Chicago, Illinois, U.S.A.) at an effective exposure index of approximately 630/29°.

Quantification of the distribution of immunofluorescence

Determinations of the distribution of MT-enrichment were made visually and with intensity profiles. Video images of light micrographs were digitized and intensity profiles derived using a Grudecard of a FOX AT-compatible computer and software developed by M. Michaelis. For the evaluation of the distribution of immunofluorescence, micrographs were printed at an indermediate density without dodging or burning in so that the relative brightness was maintained.

Results

Tip cell zonation

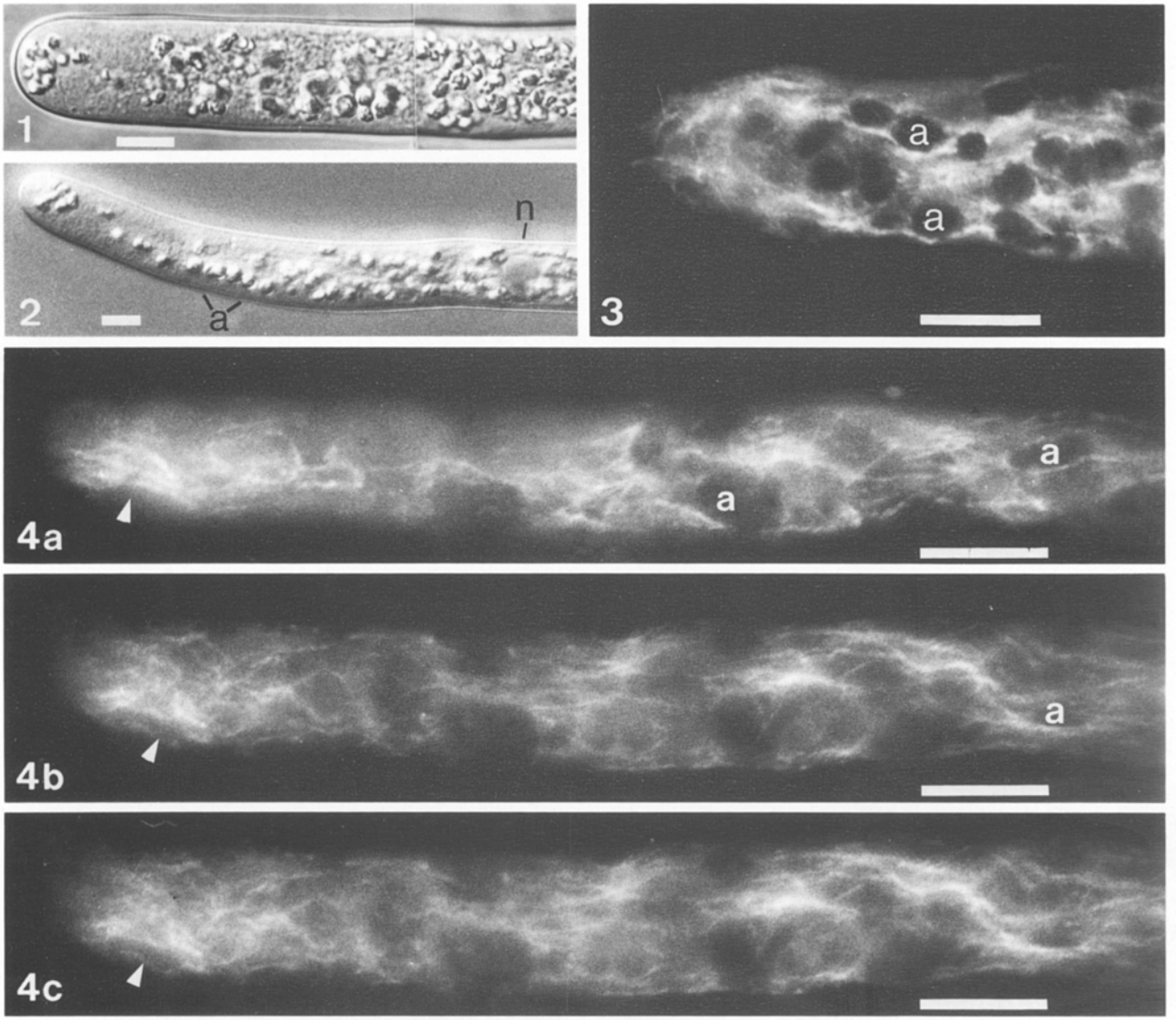
Dark-grown tip cells of *Ceratodon* protonemata have a distinct zonation that has not been described in other moss protonemata. These include four zones arranged longitudinally in series (Figs. 1 and 2). (1) The "apical dome" (approximately the first 10 μm) contains a group of 3–11 plastids. (2) A "plastid-free zone" is located 10–20 μm distal to the apical dome. (3) A "sedimentation zone" is found 20–50 μm from the tip and is the only region where significant plastid sedimentation occurs upon gravistimulation (horizontal placement). (4) The remaining distal portion of the tip cell includes a proximally-located nucleus, amylochloroplasts which do not sediment significantly, and one or more large vacuoles at the distal end of the cell.

This zonation has been described with respect to the kinetics of plastid sedimentation (Walker and Sack 1989). Initial observations of time-lapse series of curvature (using infrared videomicroscopy) indicates that vertical growth and gravitropic curvature occurs primarily in zones 1 and 2 (Young and Sack 1989). Apical cells of the protonemata are typically 250 μm long and 15 μm in diameter with a growth rate of approximately 20–25 μm/h.

General features of microtubule distribution

Microtubules (MTs) were found throughout the cytoplasm of the tip cell. They extended through all zones from the very tip to the most distal region (Figs. 5 and 11). Optical sectioning showed numerous microtubules in all planes of focus i.e. the microtubules formed a rich three dimensional network (Fig. 4a–c). Microtubules had a predominantly longitudinal orientation throughout the cell.

Microtubules extended right into the apical dome (Figs. 5 and 11). In some cases the microtubules seemed to converge in the apical dome, but it could not be



Figs. 1 and **2.** Light micrographs (DIC) of gravistimulated tip cells. Gravity vector is towards bottom of these and all following micrographs of horizontal (gravistimulated) tip cells. *a* Sedimented amylochloroplasts, *n* nucleus. Bars: 10 µm

Fig. 1. Gravitropic stimulus (GS) 30 min

Fig. 2. GS 3 h

Fig. 3. Immunofluorescence of microtubules in a tip cell gravistimulated (horizontal) for 3 h. *a* Plastids with closely associated microtubules. Bar: 10 µm

Fig. 4a–c. Serial optical sections of the same gravistimulated tip cell (GS 30 min). ▷ Position of microtubule enrichment. Most of the dark spaces contain one or more amylochloroplasts (*a*). Bar: 10 µm

determined whether these microtubules were continuous around (bend around) the dome or whether they ended (or started) in the tip zone.

Plastids were much better preserved with fixatives using PIPES buffer than with phosphate buffer; the latter treatment often resulted in broken plastids with somewhat dispersed starch grains. The apex (proximal region) of the tip cell appears to be more sensitive to cell wall digesting enzymes than the most distal regions. In prolonged enzyme treatments, the tip was dissolved (absent) but the cell wall and cytoplasm in other zones were still intact.

In immunofluorescence, both plastids and nuclei were distinguishable as darker, spherical regions surrounded

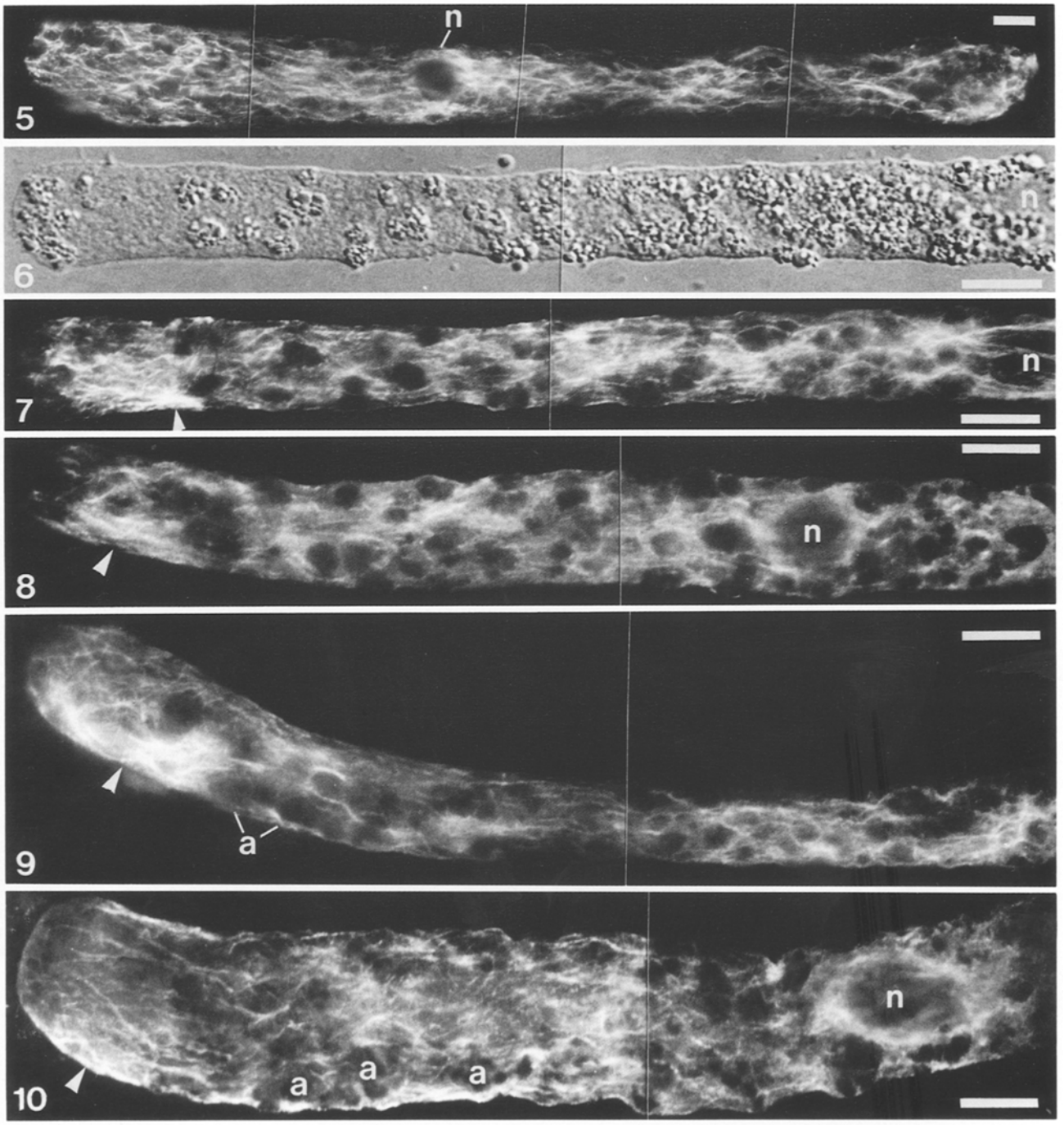


Fig. 5. Immunofluorescence of microtubules in vertical (nongravistimulated) tip cell. Apex of cell is on the left and distal (end) cell wall is on the right. Gravity vector is towards the right. Note absence of microtubule enrichment. *n* Nucleus. Bar: 10 μm

Figs. 6–10. Gravistimulated tip cells. Bars: 10 μm

Figs. 6–7. Same cell shown in DIC and immunofluorescence

Figs. 7–10. Immunofluorescence. ▷ Position of apparent microtubule enrichment after gravistimulation. Note upward gravitropic curvature in Figs. 8–10. Time gravistimulated = 45 min (Fig. 7), 1 h (Fig. 8), 3 h (Fig. 9), and 5 h (Fig. 10). *a* Sedimented amylochloroplasts, *n* nucleus

by microtubules. The identification and boundaries of these organelles were confirmed in conjugate DIC images of the same field (e.g., Figs. 6 and 7). Microtubules appeared closely associated with plastids in material prepared for immunofluorescence (Figs. 3, 10, and 14). This positional association was confirmed by optical sectioning (Fig. 4a–c).

Immunofluorescence was particularly bright around

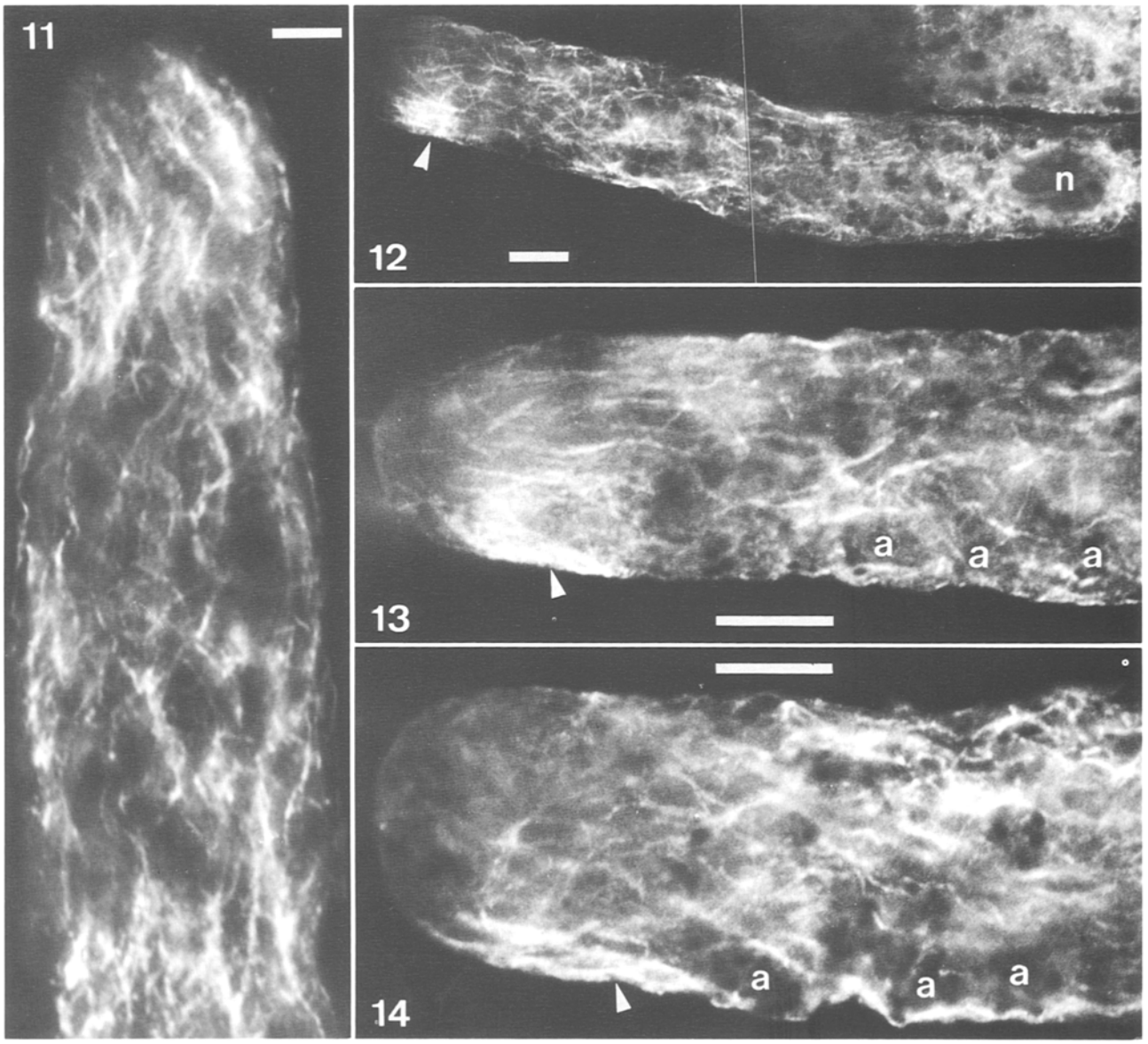


Fig. 11. Microtubule immunofluorescence of a vertical (nongravistimulated) protonema tip. Scale bar: 5 µm

Figs. 12–14. Microtubule immunofluorescence of gravistimulated protonemata (5 h). Arrowheads indicate position of microtubule/immunofluoresence enrichment. Note the proximity of microtubules to sedimented plastids *a*. *n* Nucleus. Bars: 10 µm

the interphase nucleus. Microtubules appeared to "focus" on the nucleus and to be closely associated with the nuclear envelope (Figs. 5 and 10). Mitotic spindles and phragmoplasts showed a fluorescence with a much higher intensity and density than the surrounding cytoplasmic MTs and the MTs during interphase (data not shown). The two spindle poles appeared to be closely associated with surrounding cytoplasmic MTs. Cytoplasmic MTs were always present during mitosis, but may have been less abundant than in interphase.

Microtubule distribution in gravistimulated protonemata

In completely dark-grown protonemata, curvature in response to gravistimulation was detected as early as 30 min after the onset of gravistimulation (horizontal placement) in time-lapse studies of individual protonemata examined by infrared videomicroscopy (Young and Sack 1989). In populations of protonemata, curvature was not detected until after 60–90 min of continuous gravistimulation (Walker and Sack 1989).

By careful fixation and transfer of fixed protonemata to polylysine coated coverslips, the gravitropic curvature of the tip cell could be maintained throughout processing for immunofluorescence. This processing often resulted in some plasmolysis, but curvature in gravistimulated protonemata was usually preserved in both the cell wall and in the protoplast itself. The extent of curvature and percentage of all protonemata that curved appeared similar, qualitatively, in cells processed for immunofluorescence compared to untreated controls.

Microtubules were closely associated with sedimented plastids in the sedimentation zone. Often single microtubules or groups of microtubules were present below partially or completely sedimented plastids (Figs. 3, 10, and 14).

After > 20 min of horizontal placement, tubulin immunofluorescence frequently appeared enriched (brighter) and microtubules seemed to be more abundant in the lower compared to the upper part of the plastid-free zone (Figs. 4a–c, 7–10, and 12–14). Microtubules in this enriched region appeared to have the same orientation as microtubules in the flanks of vertical protonemata. This lower flank enrichment in fluorescence was found in protonemata that had not yet curved up (e.g., fixed 30 min after being reoriented to the horizontal) or were in the process of upward gravitropic curvature (e.g., fixed 2–5 h after reorientation). Approximately two thirds of protonemata gravistimulated 20 min or longer showed an enrichment in microtubules in the lower flank (Table 1). In vertically-growing protonemata, fewer than 8% exhibited a higher density of microtubules near either side wall (Table 1). The lateral extent or thickness of the region of lower flank enrichment was quantified. Of the 86 (67.2% of all 128 horizontal protonemata; see Table 1) gravistimulated protonemata with a lower flank enrichment, 21% (of the 86) had an enrichment which occupied the bottom fifth of the tip diameter, 33% the bottom third, 37% the bottom half, and 9% the bottom two thirds.

Of the protonemata gravistimulated longer than 2 h that *lacked* a lower flank enrichment (N = 25), 40% showed no upward curvature. Of the protonemata gravistimulated longer than 2 h that did show a lower flank enrichment (N = 63), only 13% showed no upward curvature.

The density of microtubules in the upper part of the plastid-free zone in gravistimulated protonemata was comparable to the density of microtubules in other regions of the protonemata. For example, sedimentation of plastids in the sedimentation zone resulted in an upper area which contained few or no plastids; this area was similar in microtubule density to the upper part of the plastid-free zone just proximal to it.

Table 1. Frequency (%) of microtubule enrichment in vertical and horizontal protonemata[a]

Orientation	Total %	Lower flank[b]	Upper flank[b]	Symmetrical enrichment[c]	No enrichment
Vertical	100 (N = 77)	6.5	7.8	39.0	46.8
Horizontal[d]	100 (N = 128)	67.2	4.7	21.9	6.3

[a] Micrographs analyzed for all protonemata with well-labelled microtubules

[b] Arbitrary left and right flanks in vertical protonemata

[c] Overall enrichment in plastid-free zone vs. other zones but no asymmetry present within zone

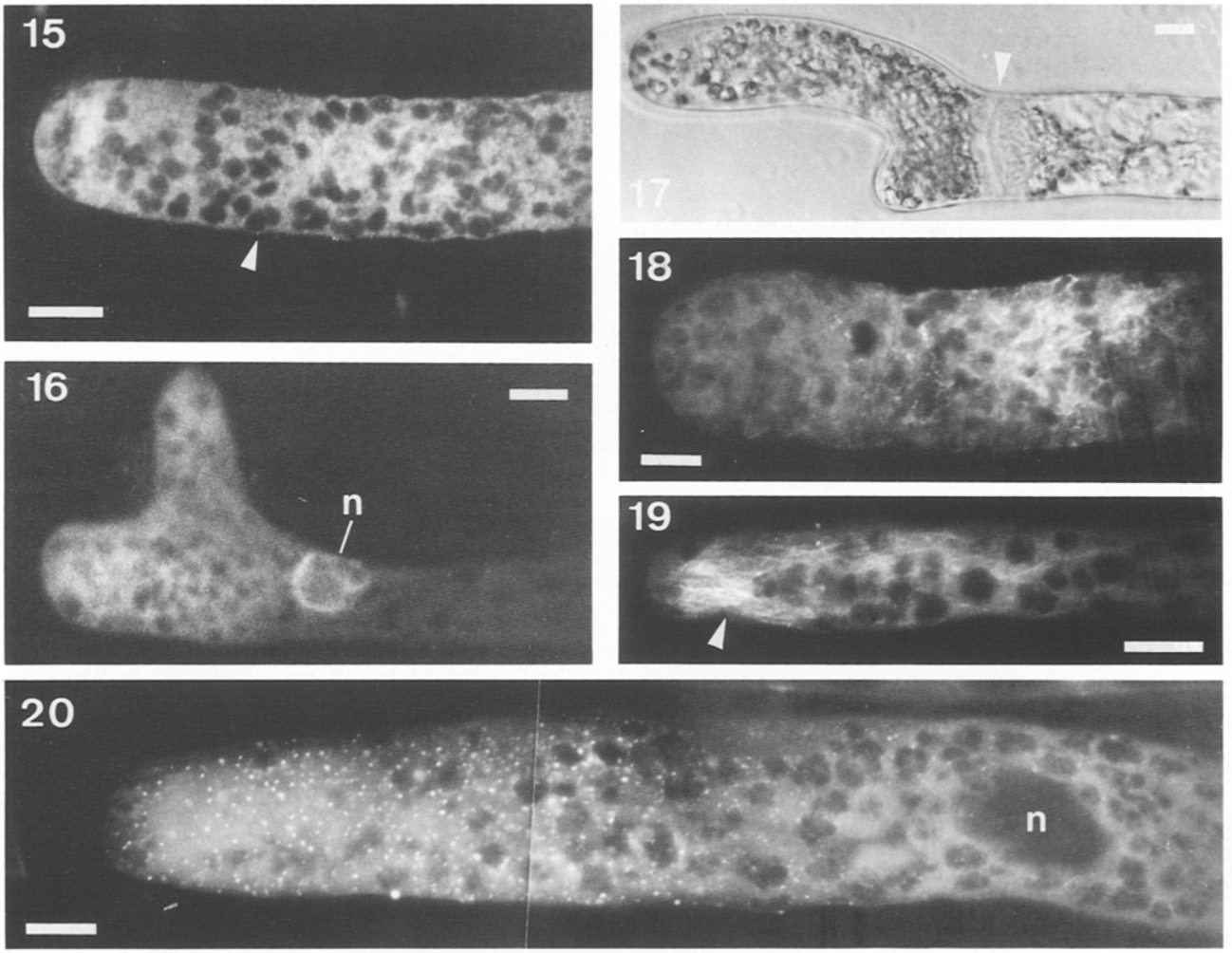
[d] Gravistimulated ⩾ 20 min

Experiments with microtubule inhibitors

Protonemata were exposed to the microtubule inhibitors oryzalin and APM in order to determine their effects on zonation, growth, microtubule distribution, and on gravicurvature.

Oryzalin

Oryzalin was primarily used at final concentrations of 0.1 μM and 1 μM and not washed from the protonemata (continuous application). A 10 min application of 0.1 μM oryzalin eliminated microtubules in the apical dome and in the plastid-free zone, and caused some disorganization of microtubules in and behind the sedimentation zone (Fig. 18). In some cases, punctate fluorescence was also observed in the cytoplasm. A 30 min application of 0.1 μM oryzalin eliminated most cytoplasmic microtubules throughout the tip cell except for phragmoplast and spindle microtubules. Two hours after application of 0.1 μM oryzalin, the zonation became disrupted and the shape of the tip changed. The plastid-free zone disappeared, the nucleus migrated close to the tip, and the tip began to swell or become asymmetrically deformed. Between 2 and 5 h of exposure to oryzalin, subapical lateral protrusions formed (Figs.16 and 17). These contained plastids throughout the protrusion and did not have the zonation characteristic of untreated protonemata. When



Figs.15–20. Tip cells treated with the microtubule inhibitors oryzalin and APM. *n* Nucleus. Bars: 10 μm

Fig. 15. Gravistimulation (GS) for 2 h 15 min, then further GS and treatment with 0.1 μm oryzalin for 45 min. Microtubules are depolymerized but soluble tubulin is labelled by immunofluorescence. Note slight gravitropic curvature. Some plastid sedimentation is still detectible (▷) but plastid-free zone is absent

Fig.16. Gravistimulation (GS) for 5 h (gravitropic curvature not shown), then treatment with 0.1 μM oryzalin and further GS for 3 h. Normal plastid zonation is absent and a subapical protrusion has formed. *n* Nucleus

Fig.17. Simultaneous gravistimulation and treatment with 0.1 μM oryzalin for 5 h. ▷ Forming cell wall. Oryzalin treatment prevented upward curvature, eliminated plastid zonation, promoted the formation of a new tip, but did not prevent cytokinesis

Fig.18. Immunofluorescence of vertical tip cell (no gravistimulation) that had been incubated with 0.1 μM oryzalin for 10 min. Most microtubules in the tip (at the left) have become depolymerized

Fig.19. Regeneration experiment: Treatment with 0.1 μM oryzalin for 1 h during GS, then 3 rinses with water, then transfer to fresh agar medium and further GS for 2 h. ▷ Position of microtubule/immunofluorescence enrichment after regeneration

Fig. 20. 1 μM APM for 5 min. Vertical cell (no GS). Depolymerization of microtubules is complete. *n* Nucleus

protonemata were gravistimulated after the start of exposure to oryzalin, they showed no measurable curvature.

Oryzalin-treated protonemata often showed a diffuse fluorescence that allowed visualization of plastids and nuclei; since these organelles did not fluoresce, their outlines could often be detected by the fluorescence of unpolymerized tubulin in the cytoplasm that surrounded them.

Some protonemata were gravistimulated for 1.5–2 h, and then treated with 0.1 μM or 1 μM oryzalin while maintained in a horizontal orientation. Even after 1.5 h

of oryzalin treatment, the plastids usually remained sedimented. In some cases however, zonation and sedimentation were lost and the nucleus could be observed close to the tip.

Some protonemata were gravistimulated for 5 h, and then exposed to 0.1 μM or 1 μM oryzalin for 3 h while maintained horizontally. Only diffuse low-intensity tubulin fluorescence was observed. In most cases, subapical lateral protrusions formed which contained plastids. The nucleus migrated towards the tip, and zonation and sedimentation were lost completely (Fig. 16).

Continuous application of 0.1% ethanol in distilled water as a control had no detectible influence on growth, gravitropic curvature, or microtubule distribution.

APM

The inhibitor APM was used at final concentrations of 1 μM and 3 μM and produced results similar to those with oryzalin. After 2 min and 5 min of 1 μM APM, punctate fluorescence was detected (Fig. 20). After 10 min to 2 h of 1 μM APM treatment, a diffuse low-intensity fluorescence appeared throughout the cytoplasm. After 2 min of 3 μM APM treatment, either diffuse or punctate fluorescence was detected.

When protonemata were exposed to 1 μM or 3 μM APM for 2 h, subapical lateral protrusions formed. Protonemata were maintained in a horizontal orientation throughout this APM application. The protrusions did not appear to be oriented with respect to gravity, and they grew out of both the upper and lower sides of the original protonemata. In these protrusions, the nucleus was present in the tip, and zonation and plastid sedimentation were absent. Prior to formation of the protrusions, in some cases, sedimentation and normal zonation were present in the apex of the tip cell despite APM treatment. The extent of sedimentation and zonation in the tip cell decreased significantly after protrusion formation.

Regeneration experiments

Protonemata were exposed to 0.1 μM oryzalin for 15 min to 1 h, rinsed 3 times with water, and then transferred to fresh agar medium for 2–3 h. In all regeneration experiments, gravistimulation started when the inhibitor was added and continued for the duration of culture on fresh agar (> 3 h continuous gravistimulation). During regeneration, microtubules first reappeared close to the tip and/or in the plastid-free zone. Normal zonation and sedimentation were present at the end of the full 2–3 h of regeneration period. Some protonemata began to curve after 2–3 h of regeneration. In several protonemata, MT-enrichment in the plastid-free zone was also detected after regeneration (Fig. 19). However, in regenerated protonemata the background immunofluorescence was greater and the microtubules were less distinct than in untreated protonemata, presumably since more unpolymerized tubulin remained after treatment with the inhibitor.

Discussion

Dark-grown *Ceratodon* protonemata have a plastid zonation (tip cluster, plastid-free zone, sedimentation zone) that is unusual for the moss genera whose protonemata have been described (Schmiedel and Schnepf 1980, Jenkins et al. 1986, Tewinkel and Volkmann 1987, Doonan et al. 1988, Walker and Sack 1990). However, in the distribution of microtubules in vertical protonemata, *Ceratodon* resembles other genera, i.e., the microtubules have a predominantly axial orientation and are located throughout the cytoplasm including in the apical dome (Doonan et al. 1985, 1988; Wacker et al. 1988).

Oryzalin and APM inhibit microtubule polymerization and depolymerize existing microtubules (Morejohn et al. 1987). In *Ceratodon* these inhibitors eliminated all microtubules and produced a diffuse and/or a punctate fluorescence in the cytoplasm. After prolonged exposure to these inhibitors, upward curvature did not take place, the shape of the tip became deformed, the nucleus migrated to the tip, and plastid zonation was destroyed. In addition, subapical lateral protrusions formed on both the upper and lower sides of the tip, and these protrusions were not gravitropic (did not grow up).

These effects of microtubule inhibitors are roughly equivalent to those described for *Physcomitrella* and *Funaria* protonemata (Schmiedel and Schnepf 1980, Doonan et al. 1988, Wacker et al. 1988) except that in *Ceratodon* the loss of plastid zonation was more pronounced and the effect of inhibitors on gravitropism in *Physcomitrella* was not described. Doonan et al. (1988) argue that protonematal microtubules regulate tip shape and maintain one center and vector (apex) of growth, but that actin microfilaments are necessary for tip extension per se (outgrowth).

Since oryzalin and APM disrupt plastid zonation in *Ceratodon*, microtubules appear to be involved in the longitudinal positioning of plastids in tip cells. This

conclusion is supported by immunofluorescence images of untreated protonemata that show a close positional association between microtubules and plastids in *Ceratodon* (e.g., Figs. 3, 4a–c, 10, and 14) and in *Funaria* protonemata (Wacker et al. 1988). Although processing for immunofluorescence undoubtedly affects spatial relationships, an association between plastids and microtubules has also been reported using the electron microscope in well-fixed protonemata (Tewinkel and Volkmann 1987).

If a normal zonation is present (before gravistimulation) in protonemata, microtubules are not directly necessary for plastid sedimentation since protonemata exposed to APM and then turned to the horizontal showed extensive sedimentation. With continued exposure to inhibitors, plastid sedimentation disappeared concomitantly with the loss of zonation; thus the disruption of sedimentation does not require microtubules. But since sedimentation only occurs in protonemata with normal zonation, microtubules appear to be necessary indirectly for sedimentation by maintaining zonation.

Microtubule inhibitors (colchicine, oryzalin, and APM) inhibit gravitropism but augment phototropism in *Ceratodon* tip cells. In the presence of these inhibitors, protonemata curved 90° towards unilateral light of an intensity that produced only 30° curvature without inhibitors (Hartmann 1984, Weber and Schwuchow unpubl.). Thus, microtubules do not regulate the shape and direction of tip growth in a simple or uniform manner in *Ceratodon* protonemata.

Two thirds of the protonemata that were gravistimulated for at least 20 min had an enrichment in microtubules in the lower but not in the upper part of the plastid-free zone. It is unlikely that this pattern is an artifact. Protonemata gravistimulated > 20 min that had an enrichment in the upper flank occurred as infrequently (5%) as vertical protonemata with an enrichment in either flank (8%). The lower flank enrichment in horizontal protonemata appeared in a consistent position and was more likely to be correlated with upward curvature; 87% of gravistimulated protonemata with a lower flank enrichment showed gravitropic curvature whereas 60% of gravistimulated protonemata without a lower flank enrichment curved up. Nor is the enrichment likely to result from the presence of large non-staining organelles in the upper half of the plastid-free zone, since this zone primarily contains cytoplasm during both vertical growth and upward curvature. It is also unlikely that this enrichment results from gravistimulation enhancing differentially the penetration of antibodies through the lower cell wall (e.g. by affecting the activity of cell wall digestion enzymes necessary for immunolabelling), since cells with a lower flank enrichment also had well-labelled microtubules in adjacent regions (upper flank, apical dome).

Many vertical and horizontal protonemata exhibited brighter tubulin immunofluorescence in the plastid-free zone compared to adjacent regions (apical dome or the sedimentation zones); however, in vertical protonemata these microtubules were distributed symmetrically across the zone (Table 1). The absence of large plastids from this zone probably contributes to the higher density of microtubules.

The lower flank enrichment was found after 20 min or more of gravistimulation. Plastid sedimentation in these cells can be detected within 15 min of gravistimulation in the region just distal to where microtubules become enriched (Walker and Sack 1990). Upward curvature does not start until 60–90 min of gravistimulation. Perhaps plastid sedimentation results directly or indirectly in microtubule repositioning or in enhanced tubulin polymerization which might in turn influence the growth of the more rapidly growing lower cell wall. By this reasoning, the absence of a lower flank enrichment in 33% of horizontal protonemata may indicate either that the enrichment is not essential for curvature or that it occurs transiently (e.g., absent during cytokinesis) and only contributes to curvature when present.

Acknowledgements

The Deutscher Akademischer Austauschdienst is gratefully acknowledged for support for the senior author to work at The Ohio State University.

References

Banbury GH (1962) Geotropism of lower plants. In: Bünning E (ed) Physiologie der Bewegungen: Bewegungen durch Einflüsse der Temperatur, Schwerkraft, chemischer Faktoren und aus inneren Ursachen. Springer, Berlin Göttingen Heidelberg, pp 344–377 [Ruhland W et al (eds) Handbuch der Pflanzenphysiologie – Encyclopedia of plant physiology, vol 17, part 2]

Bartnik E, Sievers A (1988) In-vivo observations of a spherical aggregate of endoplasmic reticulum and of Golgi vesicles in the tip of fast-growing *Chara* rhizoids. Planta 176: 1–9

Doonan JH, Cove DJ, Lloyd CW (1985) Immunofluorescence microscopy of microtubules in intact cell lineages of the moss, *Physcomitrella patens*. I. Normal and CIPC-treated cells. J Cell Sci 75: 131–147

– – Corke FMK, Lloyd CW (1987) Pre-prophase band of microtubules, absent from tip-growing moss filaments, arises in leafy shoots during transition to intercalary growth. Cell Motil Cytoskeleton 7: 138–153

– – Lloyd CW (1988) Microtubules and microfilaments in tip growth: evidence that microtubules impose polarity on protonemal growth in *Physcomitrella patens*. J Cell Sci 89: 533–540

Hartmann E (1984) Influence of light on phototropic bending of moss protonemata of *Ceratodon purpureus* (Hedw.) Brid. J Hattori Bot Lab 55: 87–98

– Klingenberg B, Bauer L (1983) Phytochrome-mediated phototropism in protonemata of the moss *Ceratodon purpureus* Brid. Photochem Photobiol 38: 599–603

Jenkins GI, Courtice GRM, Cove DJ (1986) Gravitropic responses of wild-type and mutant strains of the moss *Physcomitrella patens*. Plant Cell Environ 9: 637–644

Kiss JZ, Sack FD (1989) Reduced gravitropic sensitivity in roots of a starch-deficient mutant of *Nicotiana sylvestris*. Planta 180: 123–130

Morejohn LC, Bureau TE, Mole-Bajer J, Bajer AS, Fosket DE (1987) Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. Planta 172: 252–264

Sack FD, Kiss JZ (1989) Plastids and gravity perception. In: Boyer CD, Shannon JC, Hardison RC (eds) Physiology, biochemistry, and genetics of nongreen plastids. American Society of Plant Physiologists, Rockville, Maryland, pp 171–181

Schmiedel G, Schnepf E (1979) Side branch formation and orientation in the caulonema of the moss, *Funaria hygrometrica*: normal development and fine structure. Protoplasma 100: 367–383

– – (1980) Polarity and growth of caulonema tip cells of the moss *Funaria hygrometrica*. Planta 147: 405–413

Schnepf E (1986) Cellular polarity. Annu Rev Plant Physiol 37: 23–47

Tewinkel M, Volkmann D (1987) Observations on dividing plastids in the protonema of the moss *Funaria hygrometrica* Sibth. Planta 172: 309–320

Volkmann D, Sievers A (1979) Graviperception in multicellular organs. In: Haupt W, Feinleib ME (eds) Physiology of movements. Springer, Berlin Heidelberg New York, pp 573–600 [Pirson A, Zimmermann MH (eds) Encyclopedia of plant physiology, ns, vol 7]

Wacker I, Quader H, Schnepf E (1988) Influence of the herbicide Oryzalin on cytoskeleton and growth of *Funaria hygrometrica* protonemata. Protoplasma 142: 55–67

Walker LM, Sack FD (1990) Amyloplasts as possible statoliths in gravitropic protonemata of the moss *Ceratodon purpureus*. Planta 181: 71–77

White RG, Sack FD (1990) Actin microfilaments in presumptive statocytes of rootcaps and coleoptiles. Amer J Bot 77: 17–26

Young JC, Sack FD (1989) Videomicroscopy-based observation of gravitropism in individual protonema of the moss *Ceratodon*. Am Soc Gravitat Space Biol Bull 2: 61