Microtubules and microfilaments in tip growth: evidence that microtubules impose polarity on protonemal growth in *Physcomitrella patens*

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Summary

In this study we compare the contributions of F-actin and microtubules to tip growth in filamentous cells of the moss *Physcomitrella patens*.

In tip growth, expansion seems to be restricted to the hemispherical apical dome. Cytoskeletal elements have been suspected, from drug studies, to be involved in this but electron microscopy has generally not confirmed the presence of an apical cytoskeleton. However, in a previous immunofluorescence study we reported that microtubules could be seen to focus upon the apical dome in tip cells of the moss *P. patens*. In the present investigation F-actin has also been detected at the apices of these cells. Anti-cytoskeletal drugs were therefore used to differentiate between the roles of actin filaments and microtubules in tip growth.

At high concentrations (30 μM), the herbicide cremart de-polymerized microtubules and caused tip swelling. F-actin was still present under such conditions but its fragmentation by cytochalasin D suppressed this herbicide-induced swelling. On its own, cytochalasin D arrested tip growth without causing swollen tips.

At lower concentrations, cremart disorganized microtubules rather than causing their complete depolymerization. Under these conditions, new but swollen growing points were initiated along the filament. The addition of taxol to cremart-treated filaments tended to reduce swelling and to re-polarize outgrowth. With particular combinations of these drugs, multiple lateral outgrowths were initiated in the vicinity of the nucleus.

It is concluded: (1) that F-actin is present at the tips of *Physcomitrella* caulonemal apical cells; (2) that unfragmented F-actin is necessary for outgrowth; (3) that even disorganized microtubules permit some degree of outgrowth but that an unperturbed distribution of axial microtubules, focussing upon an apex, is essential in order to impose tubular shape and directionality upon expansion.

Key words: microtubules, microfilaments, tip growth, anti-cytoskeletal drugs, moss.

Introduction

Restriction of cell expansion to a local specialized zone of the cell cortex is the characteristic mode of growth displayed by a wide range of filamentous, tip-growing cells. This process requires that materials necessary for growth are transported and delivered with some precision (Schnepf, 1986). The polarized exocytosis, within the apical dome, of Golgi-derived vesicles is thought to provide membrane (Picton & Steer, 1983), cellulose-synthesizing complexes, cell wall matrix precursors (Reiss *et al.* 1984), and ion pumps and channels (Brawley & Robinson, 1985) necessary for cellular extension. How these materials are propelled and guided to the tip is not clear but drug inhibition studies implicate both microtubules (Schnepf *et al.* 1985) and microfilaments (Seagull & Heath, 1980; Picton & Steer, 1982).

Understanding the mechanism of tip growth will undoubtedly help in interpretation of the process by which apical cells respond to directional changes in environmental stimuli. For instance, protonemata of the moss *Physcomitrella patens* bend with respect to a unidirectional light source and to gravity (Cove *et al.* 1978).

Immunofluorescence studies have shown that apical caulonemal cells of *P. patens* contain microtubule arrays that extend into the apical dome where they converge to form foci (Doonan *et al.* 1985). As for actin filaments, although they have been detected in root hairs (Seagull & Heath, 1979; Lloyd *et al.* 1987) and pollen tubes (Perdue & Parthasarathy, 1985; Pierson *et al.* 1986), they have not been seen in moss caulonemata.

In this study, we have detected F-actin in these tip-growing cells. To differentiate between the roles of F-actin and microtubules in tip growth, selective drugs have been used and their effects monitored by fluorescence microscopy. Because colchicine is now known to be far less effective at de-polymerizing plant than animal microtubules (Morejohn & Fosket, 1984*a*), cremart was used instead. This herbicide effectively de-polymerizes microtubules in various plant cells (Doonan *et al.* 1986; Lloyd *et al.* 1987). Particular attention was paid to possible cytoskeletal involvement in: (1) confinement of growth to the tip; (2) maintenance of a constant cell diameter; and (3) maintenance/alteration of the direction of cell elongation.

It is concluded that while microfilament integrity is essential for growth *per se*, microtubules are the components that control the direction of cell expansion, and the tubular morphology of caulonemata.

Materials and methods

Materials

Moss protonemata were grown on Knop's medium overlaid by cellophane as described (Doonan *et al.* 1985). Using the underlying cellophane as a support, plants were transferred on to medium containing drugs. Stock solutions of the anti-microtubule herbicide cremart (gift from the Sumitomo Chemical Co. Ltd, Takatsukasa, Takarazuka, Hyogo, Japan), taxol (gift from Dr M. Suffness, National Cancer Institute, Bethesda, Maryland, USA), and cytochalasin D (Sigma) were prepared at 100 times their final concentration, using dimethylsulphoxide (DMSO) as a solvent. Where mixtures of more than one drug were used, a stock solution containing both drugs was prepared. Control plants were transferred to growth media containing 1% (v/v) DMSO, which produced none of the effects observed on adding drugs.

Indirect immunofluorescence of microtubules

Fixation, permeabilization and staining were essentially as described (Doonan *et al.* 1985), except that anti-tubulin YOL 1/34 was used in addition to YL 1/2 (Kilmartin *et al.* 1981).

Visualization of microfilaments with rhodaminyl lysine phallotoxin

Protonemata were immersed in 2% (w/v) Driselase for 5 min, then rinsed in microtubule-stabilizing buffer (MTSB: 100 mM-Pipes, pH 6·8, 5 mM-EGTA, 5 mM-$MgSO_4$) and lysed in MTSB containing 10% (v/v) DMSO, 0·1% (v/v) Nonidet NP40 for 2–5 min. Rhodaminyl lysine phallotoxin (a gift from Professor Wieland, Max Planck Institute, Heidelberg) and DAPI were added to final concentrations of 0·5 $\mu g\,ml^{-1}$ and 1·0 $\mu g\,ml^{-1}$, respectively, and observed after a further 10 min using Zeiss epifluorescence optics. Controls incubated in unlabelled phalloidin (5 $\mu g\,ml^{-1}$) for 30 min prior to staining with rhodaminyl lysine phallotoxin eliminated specific staining of microfilaments.

Results

Organization of the cytoskeleton within tip cells

Under Nomarski optics the apical dome of caulonemal cells is usually devoid of large organelles. This produces the effect of a clear cytoplasmic zone (Fig. 1A). Within this zone the approximately axial cytoplasmic microtubules converge to form foci as shown by indirect immunofluorescence (Fig. 1B).

An extensive array of actin microfilaments is found when gently lysed cells are stained with rhodaminyl lysine phallotoxin (Fig. 1C). Bundles of actin filaments weave amongst the chloroplasts, approximately along the axis of the cell (as is the case with microtubules) but fewer in number. The microfilament arrays are extremely sensitive to aldehyde fixation, being completely eliminated by a 5-min treatment with 1% formaldehyde (Traas & Doonan, unpublished observations). Thus double staining for microtubules and F-actin has not been possible.

Cremart-induced microtubule disassembly

Two cytoskeletal systems are therefore detected within the apical region and selective drugs were used to study their relative contributions to tip growth.

Cremart has been previously shown to cause complete, rapid and reversible disassembly of microtubules in sub-apical cells of *Physcomitrella* (Doonan *et al.* 1986). The apical cell is also very sensitive to this substance, but here we have used a range of concentrations designed to compare the effects on cell morphology of complete removal of microtubules with those of partial disassembly.

The effects of cremart treatment follow this time course: cell shape is unchanged after 1 h in 1 μM-cremart even though immunofluorescence reveals extensive disassembly of microtubules in the apical dome (Fig. 2A). Microtubules closer to the nucleus are less sensitive to the herbicide. The nuclear outline is

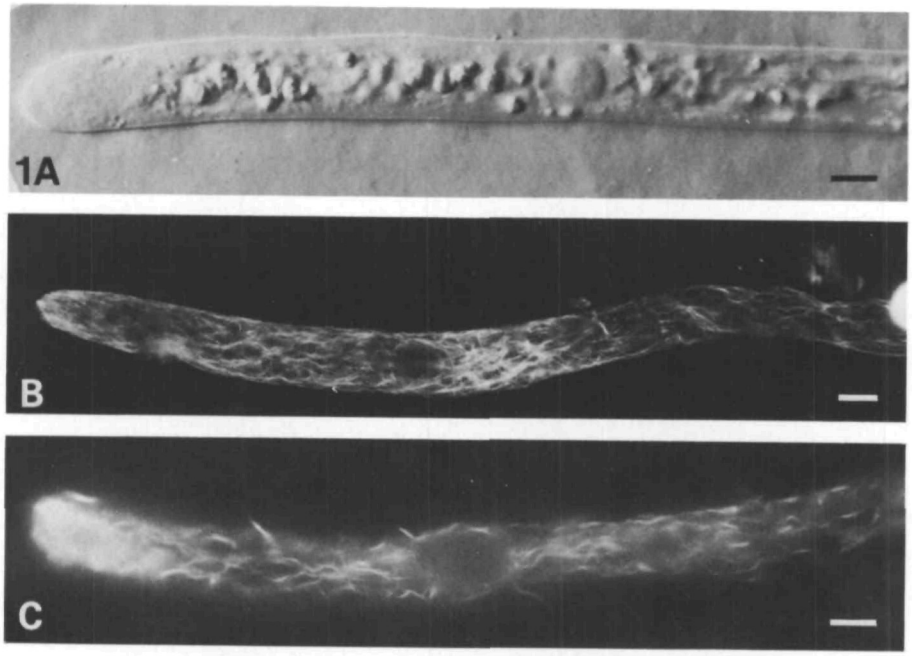

Fig. 1. The cytoskeleton of the apical caulonemal cell of *P. patens*. A. Nomarski optics show the presence of a clear zone at the tip, in which large inclusions are absent. B. Indirect immunofluorescence using anti-tubulin demonstrates the net axial microtubules that focus at a point at the very apex of the cell. C. An extensive network of microfilaments can be detected when permeabilized cells are stained with rhodamine phalloidin. They are net axial and are present within the apical dome. Bars, 10 μm.

prominent in these cells due to its association with numerous fragments of microtubules.

By 3–4 h in 1 μM-cremart, swellings appear along the flanks of apical cells. Microtubule arrays remain fragmented, although traces of organization can be perceived (Fig. 2B,C). Where bulges have formed, clumps of fragmented microtubules are found as loose foci (Fig. 2C).

By 12–14 h in 1 μM-cremart, the majority (90–95 %) of tip cells possess either a bent tip (Fig. 2D) or lateral bulges, which have begun to grow out. Microtubules are present within such protrusions, but appear to be disorganized (Fig. 2D).

Such lateral protrusions are almost completely suppressed by raising the cremart concentration to 10 μM and completely so at 30 μM. The major morphological effect of the higher concentration is to produce a swelling of the cell's tip (Fig. 3A,B). At these higher concentrations of herbicide, no microtubules are detectable either after short (Fig. 3A), or 24-h long, incubation (Fig. 3B).

Effect of cremart on microfilaments

Microfilaments remain throughout cremart treatment and are found even in swollen tips treated with 30 μM herbicide for 24 h (Fig. 3C).

Effect of taxol

Taxol, at 30–40 μM, completely inhibits further cell elongation, although the apex may swell slightly (Fig. 4A). The cytoplasm appears very granular, with numerous small vacuoles present. Lower concentrations of taxol (10 μM) slow tip growth, and occasionally induce swellings and protuberances to the rear of the apical dome (Fig. 4B). The cytoplasmic structure of taxol-induced protuberances differs from that of cremart, in that the demarcation between the clear zone and the organelle-containing cytoplasm is more pronounced in the former. The clear zone is also enlarged, and the dome itself more variable in profile; those shown in Fig. 4B are pointed, but others can be flattened. The nucleus may eventually (after 24–48 h) migrate to the base of the new 'filament', where it may become straddled between the two tips. Microtubule arrays become bundled into thick cables, and the apical foci appear to be smaller but more numerous, giving the effect of increased brightness around the rim of the apical dome (Fig. 4C). A similar image is obtained from cells treated with 40 μM-taxol, but the surface of the swollen apex is very densely stained (Fig. 4D).

Synergistic effects of cremart and taxol on induction of new sites of tip growth

The microtubule-stabilizing agent taxol has been shown partially to repair cremart-induced microtubule disassembly, which would otherwise inhibit nuclear migration in *sub-apical* cells of *Physcomitrella* (Doonan *et al.* 1986). However, when applied simultaneously to apical cells these oppositely acting compounds induce a more prolific production of new sites of tip growth. This is best demonstrated by using intermediate concentrations of both cremart (10 μM) and taxol (5 μM) (Fig. 5A). The cell apex becomes deformed and there is evidence of bending during growth, as well as of numerous small accessory apices near the nucleus. Higher concentrations of cremart (30 μM) in combination with taxol (5 μM) induce swellings that form along the flank of the apical dome (Fig. 5B), some of which may elongate (Fig. 5C).

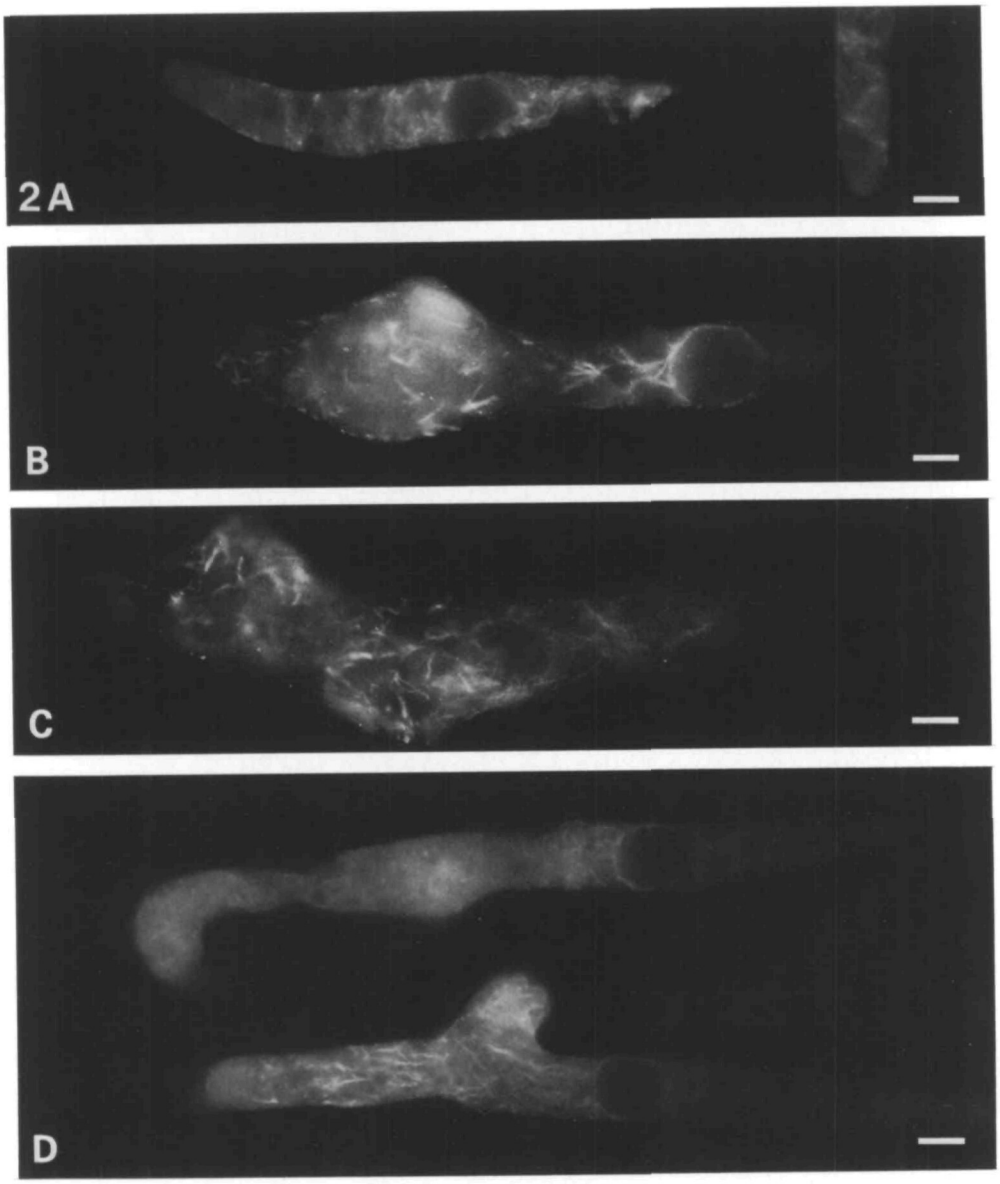

Fig. 2. The effects of the anti-microtubule herbicide cremart (at 1 μM) upon apical microtubules. A. Anti-tubulin immunofluorescence shows that after 60 min in herbicide, microtubules are absent from the tip but fragments remain in the vicinity of the nucleus. After 3–4 h (B,C) the tip cells begin to swell. Disorganized bundles of microtubules occur in the swellings. D. After 14 h treatment the microtubular arrays are more extensive than after brief treatment with herbicide. The abnormal lateral protrusions that develop contain microtubules (lower cell), albeit in a disorganized manner. Bars, 10 μm.

Immunofluorescent images of these cells reveal poorly preserved, very fragmented, but nonetheless extensive, arrays of short microtubules, which are present in all the arms of the cell (data not shown). Taxol therefore partly overcomes the effects of cremart to permit some degree of filamentous growth.

Effect of cytochalasin on microtubules and microfilaments

Cytochalasin up to 50 μg ml^{-1} had no effect upon microtubular organization but did fragment the microfilament system (not shown).

No concentration of cytochalasin B or D was found that caused new sites of tip growth. At concentrations of cremart that permit tip swelling, the simultaneous addition of cytochalasin suppressed this morphological effect.

Discussion

Electron-microscopic studies, using conventional fixatives and buffers, have failed to reveal a cytoskeletal system within the apical dome of a variety of tip-growing cells, including moss protonemata (Doonan &

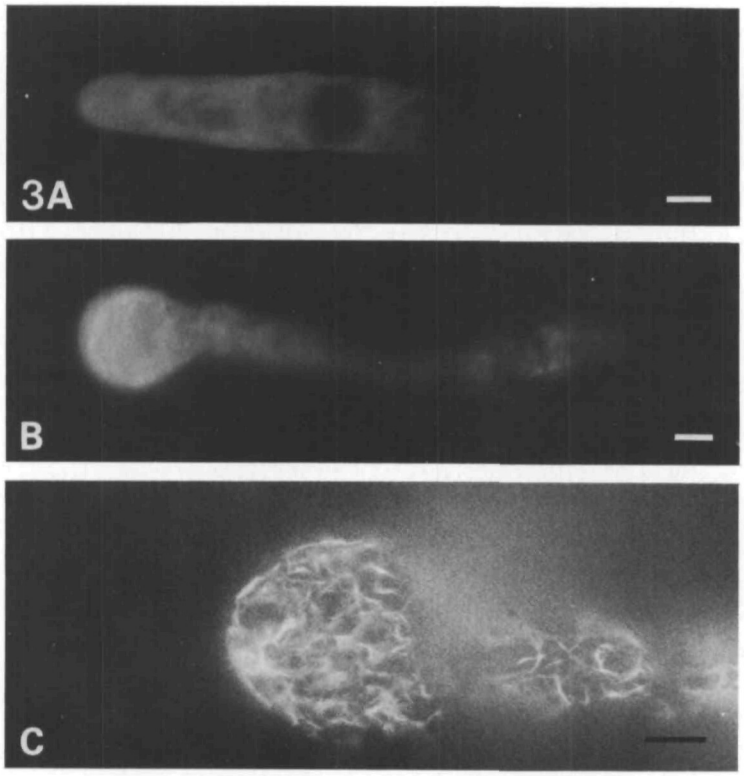

Fig. 3. Immunofluorescence microscopy shows that higher concentrations (30 μM) of cremart cause complete disassembly of microtubules. After 60 min (A) no microtubules can be detected. Neither do they re-assemble over a 24-h period. The long-term effect of such depolymerization is to produce a swollen tip (B). Although microtubules are absent from swollen tips (B), F-actin can be detected using rhodamine phalloidin (C). Bars, 10 μm.

Clayton, 1987). Thus the involvement of the cytoskeleton in tip growth has been questionable (see Schnepf, 1986), even though microtubule-perturbing drugs cause dramatic abnormalities in the growth of protonemata (Schnepf, 1983).

However, the recent use of microtubule-stabilizing buffers during fixation, combined with indirect immunofluorescence, has clearly demonstrated microtubules at the very tips of a range of moss protonemata (Doonan *et al.* 1985; Doonan & Duckett, unpublished data). Such techniques have now revealed microtubules extending into the tips of root hairs (Lloyd, 1983; Lloyd & Wells, 1985), pollen tubes (Pierson *et al.* 1986) and fungal hyphae (Hoch & Staples, 1983; Runeberg *et al.* 1986).

F-actin has been detected entering the tips of aldehyde-fixed root hairs (Lloyd *et al.* 1987). This approach was not, however, successful here and F-actin could be detected in moss only by avoiding aldehyde and using, instead, the detergent extraction method developed for carrot suspension cells (Traas *et al.* 1987).

As to the respective contributions of these two cytoskeletal systems to tip growth, the functional integrity of both appears to be essential for normal cell elongation. However, results of selective drug perturbation studies show that actin filaments and microtubules play different parts. Cytochalasin treatment, which fragments actin cables, arrests tip growth but does not produce tip abnormalities (see also Lloyd *et al.* 1987). This contrasts with the effects of the microtubule-targeted agents, cremart and taxol, which do not arrest tip growth but do produce abnormal patterns of apical growth. Under conditions (30 μM-cremart) where microtubules but not microfilaments are de-polymerized, the tip swells. Since the effect of cytochalasin D is to suppress swelling, it can be concluded that microfilaments are essential for continued apical protrusion but that microtubules govern the pattern of outgrowth, thus maintaining constant filament diameter. This requirement for both cytoskeletal elements is consistent with other recent findings. Menzel & Schliwa (1986) found that both were necessary for directional movement of chloroplasts in the green alga *Bryopsis*; Lloyd *et al.* (1987) reported that both were essential for tip growth of *Vicia hirsuta* root hairs.

At lower levels of cremart, microtubules are not completely depolymerized but their organization is altered and additional outgrowths are induced to form. Again this suggests that microtubules are required for outgrowth, even in abnormal protrusions.

Because taxol was shown partially to restore nuclear migration to cremart-treated sub-apical cells of *Physcomitrella* (Doonan *et al.* 1986), the microtubule-stabilizing agent was used here in an attempt to overcome the negative effects of cremart on tip growth. Morejohn & Fosket (1984*b*) showed that the related herbicide, amiprophos-methyl, directly poisoned the taxol-induced polymerization of rose tubulin *in vitro*.

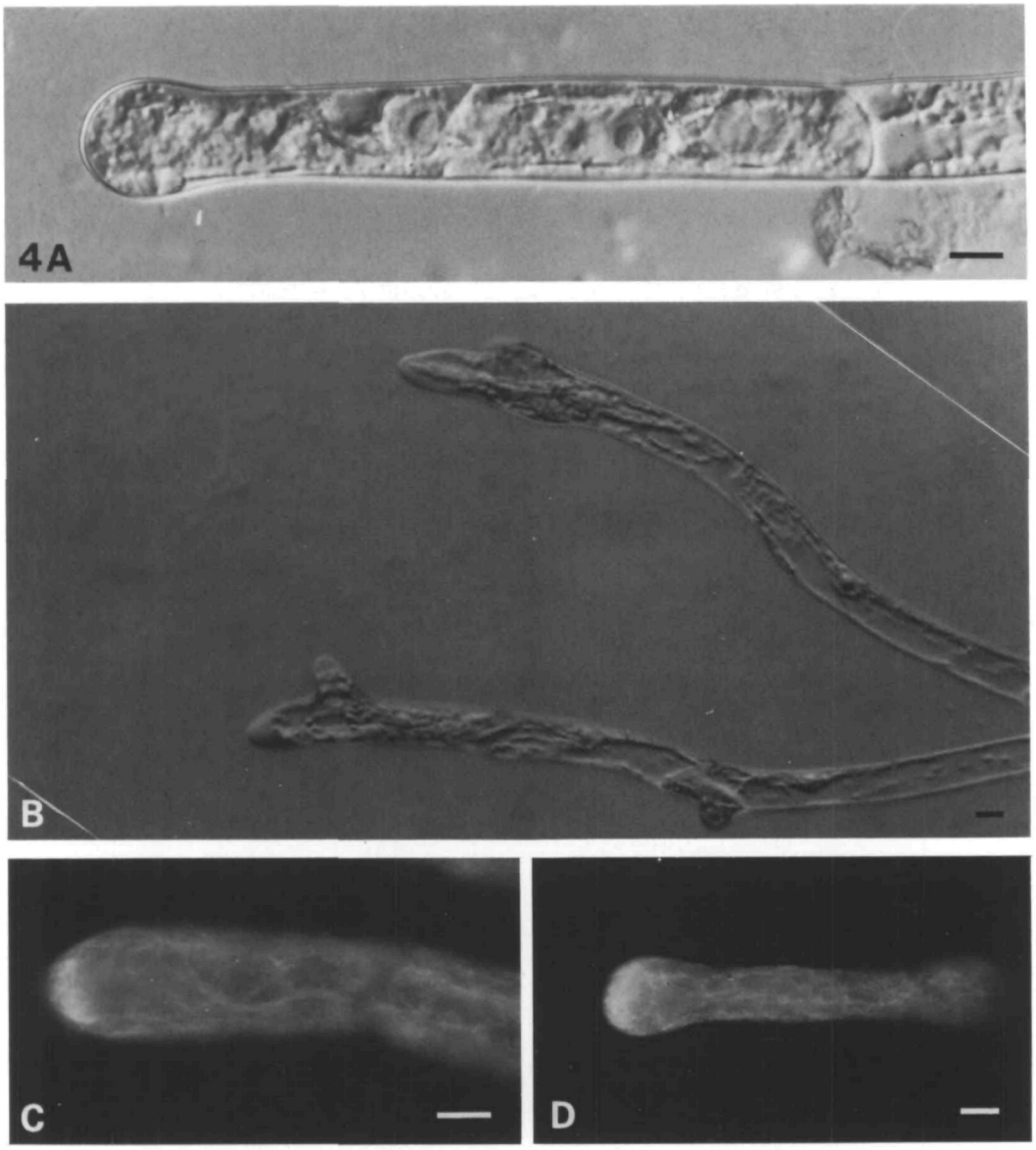

Fig. 4. Effects of taxol on tip growth. Overnight incubation (14 h) in 40 μM-taxol causes the apex to swell and further growth ceases (A). Lower concentrations (10 μM) can change tip morphology; swellings and extra tips are also induced (B). Anti-tubulin staining of the apical dome is increased by both 10 μM (C) and 40 μM (D) taxol. Bars, 10 μm.

It is likely, therefore, that taxol and cremart, respectively, shift the balance between assembly and disassembly in a similar manner and that this is the basis of their effects *in vivo*. In the moss, 5 μM-taxol does overcome the inhibitory effect of high levels of cremart on tip growth. More notably, when lower levels of cremart are used, multiple new sites of outgrowth are promoted by taxol and these new tips form in the vicinity of the nucleus. Similar observations have been made before. Schmiedel & Schnepf (1980) reported that new outgrowths formed near the nuclei of the related moss, *Funaria*, 6–10 h after colchicine treatment. Mizukami & Wada (1983) also reported that multiple side branches formed during recovery of the alga *Bryopsis plumosa* from treatment with colchicine, vinblastine or griseofulvin. Infection by *Rhizobium* can also induce legume root hairs to develop side branches, in which case they grow in the vicinity of the nucleus (Nutman, 1959). We interpret this according to the following scheme. Human sera directed against amorphous pericentriolar material have been used to locate the equivalent microtubule nucleation sites in plants (Clayton *et al.* 1985). In this case, the sites were located around the nucleus. The observation that cremart induces disassembly of microtubules at the tips of moss filaments, but that microtubules in the vicinity of the nucleus are more resistant, suggests a similar location of nucleation sites for moss; the ends of microtubules distal to nucleation sites generally being the ends that depolymerize. Normally, the apically directed cytoplasmic

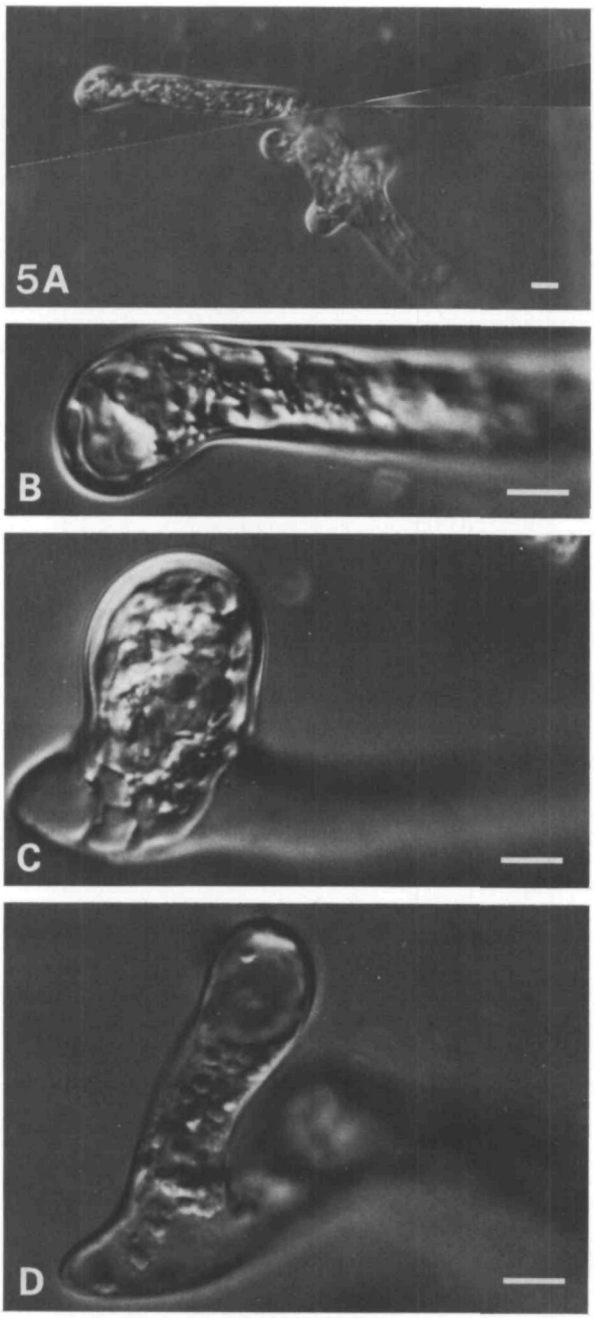

Fig. 5. Taxol induces the recovery of anomalous tip growth in cremart-treated cells. At low levels of cremart (10 μM) the presence of 5 μM-taxol can induce additional outgrowths in the vicinity of the nucleus (A). At higher levels of cremart (30 μM) tips normally swell and stop growing (see Fig. 3B). Addition of taxol, however, causes the initially swollen tip (B) to resume filamentous growth over a 30-h period (C,D). Bars, 10 μm.

microtubules in these tip cells do not undergo bouts of depolymerization (Doonan *et al.* 1985); unlike those in higher plant cells, the cytoplasmic microtubules at the apices of moss filaments persist throughout mitosis and cytokinesis, representing a useful mechanism for the preservation of apical dominance. But drug-induced depolymerization over-rides this natural persistence of apical microtubules and we interpret the new lateral outgrowths (particularly those stimulated by added taxol) to be the result of microtubule re-growth from perinuclear sites of assembly.

In a variety of animal cells, directional outgrowth depends upon directional transport of vesicles from the Golgi apparatus. This, in turn, depends upon unperturbed microtubules. Both disassembly of microtubules (see Thyberg & Moskalewski, 1985, for review) and taxol treatment (Wehland *et al.* 1983; Rogalski & Singer, 1984) cause the Golgi to fragment and become dispersed. Similarly for *Funaria*, the colchicine treatment that induces formation of novel sites of tip growth causes dictyosomes to become dispersed (Schmiedel & Schnepf, 1980).

Considering the evidence as a whole, we conclude that microfilaments are responsible for propelling growth materials to the tip, but that the necessary vectorial information, and the limitation of growth to a single apical focus, are provided by net axial microtubules, which progress from the nuclear region and focus upon the distal tip. Where microtubules become de-focussed, insertion of new growth materials continues, but over a larger area inconsistent with tubular growth.

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