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ORIGINAL ARTICLE

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Microtubules and microfilaments coordinate to direct a fountain streaming pattern in elongating conifer pollen tube tips

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Abstract This study investigates how microtubules and microfilaments control organelle motility within the tips of conifer pollen tubes. Organelles in the 30-μm-long clear zone at the tip of *Picea abies* (L.) Karst. (Pinaceae) pollen tubes move in a fountain pattern. Within the center of the tube, organelles move into the tip along clearly defined paths, move randomly at the apex, and then move away from the tip beneath the plasma membrane. This pattern coincides with microtubule and microfilament organization and is the opposite of the reverse fountain seen in angiosperm pollen tubes. Application of latrunculin B, which disrupts microfilaments, completely stops growth and reduces organelle motility to Brownian motion. The clear zone at the tip remains intact but fills with thin tubules of endoplasmic reticulum. Applications of amiprophosmethyl, propyzamide or oryzalin, which all disrupt microtubules, stop growth, alter organelle motility within the tip, and alter the organization of actin microfilaments. Amiprophosmethyl inhibits organelle streaming and collapses the clear zone of vesicles at the extreme tip together with the disruption of microfilaments leading into the tip, leaving the plasma membrane intact. Propyzamide and oryzalin cause the accumulation of membrane tubules or vacuoles in the tip that reverse direction and stream in a reverse fountain. The microtubule disruption caused by propyzamide and oryzalin also reorganizes microfilaments from a fibrillar network into pronounced bundles in the tip cytoplasm. We conclude that microtubules control the positioning of organelles into and within the tip and influence the direction of streaming by mediating microfilament organization.

Keywords Cytoskeleton · Microfilament · Microtubule · Motility · *Picea* · Pollen tube

Abbreviations APM: Amiprophosmethyl · FITC: Fluorescein isothiocyanate · LATB: Latrunculin B

Introduction

Pollen tubes deliver sperm cells to the egg for fertilization in higher plants and are an established model system for examining polarized cell growth. Vesicle fusion at the pollen tube tip drives elongation (reviewed in Hepler et al. 2001). This process is dependent on the influx of extracellular calcium at the tip (Pierson et al. 1996), the electric current established by the influx of protons at the tip and efflux along the tube shank (Feijo et al. 1999), the activity of RhoGTPases (Chen et al. 2003), the microfilament cytoskeleton and cytoplasmic streaming (Miller et al. 1996). In rapidly elongating angiosperm pollen tubes, organelles move in a reverse fountain pattern, using myosin to move along microfilaments. Organelles move in the cell cortex towards the growing tip, turn and move away from the tip in the center of the tube (Taylor and Hepler 1997; Hepler et al. 2001). Elongation and cytoplasmic streaming are sensitive to alterations in microfilament organization (Heslop-Harrison and Heslop-Harrison 1989; Gibbon et al. 1999) and there is a rapid turnover of microfilaments controlled by actin-binding proteins in the tube apex (Vidali et al. 2001; Chen et al. 2002). Microtubules are absent from the pollen tube apex (Lancelle and Hepler 1992; Pierson et al. 1986) and their disruption has little effect on elongation in angiosperm pollen tubes (Heslop-Harrison et al. 1988; Åström et al. 1995).

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Conifer pollen tubes grow much slower than their angiosperm counterparts, with rates in culture of 20 μm h^{-1} for *Picea abies* (Norway spruce, Pinaceae) compared to 5–25 μm min^{-1} in culture for flowering plants (Pierson et al. 1996; Parton et al. 2001). The conifer pollen tube must elongate through several millimeters of megagametophyte tissue to reach the egg cell and since the spermatogenous cells initially remain within the pollen grain, callose plugs cannot form to isolate the elongating apical region of the tube (Dawkins and Owens 1993; Runions and Owens 1999). Elongation is dependent not only on microfilaments and myosin, but also on an intact microtubule cytoskeleton (Anderhag et al. 2000). Organelles do not typically stream in a reverse fountain pattern in conifer pollen tubes. Instead the dominant pattern in *Pinus sylvestris* pollen tubes is a fountain, with organelles moving towards the tip in the tube center and away from the tip along the cell cortex (de Win et al. 1996). The tip does not contain an inverted cone of secretory vesicles. Instead a clear zone lacking amyloplasts but enriched in mitochondria and the endomembrane system extends 20–30 μm back from the tip. There is a demarcation running perpendicular to the tube axis between this clear zone and the amyloplasts in the rest of the pollen tube (de Win et al. 1996; Lazzaro 1996).

In conifer pollen tubes, microfilaments and microtubules form a dense network that emanates from within the pollen grain and extends in an axial array through the length of the tube (de Win et al. 1996; Lazzaro 1996, 1999). Microfilaments extend into the back of the clear zone and are found sparingly within the extreme tip of live cells (Anderhag et al. 2000). However, microtubules extend into the extreme apex of the elongating tip. The microtubules are in an axial array within the center of the tip while microtubules directly beneath the plasma membrane at the tip are organized in a net radial array that extends from the base of the clear zone to the tip (Lazzaro 1999). Microtubule disruption inhibits pollen tube elongation and causes tip bifurcation (Anderhag et al. 2000). The loss of the radial array causes specific tip swelling (Anderhag et al. 2000) and this array is linked to the deposition of cellulose microfibrils at the extreme tip (Lazzaro et al. 2003). It is intriguing that the clear presence of microtubules in the tip and the increase in branching and obvious swelling induced by their absence is completely different from angiosperm pollen tubes but resembles tip growth in protonema cells of mosses (Schwuchow et al. 1990) and ferns (Kadota et al. 1999).

This study investigates how microfilaments and microtubules coordinate the unusual fountain pattern in conifer pollen tubes. Our hypothesis is that the unusual fountain streaming pattern in conifer pollen tubes is derived from differences in cytoskeletal organization between angiosperms and conifers. By disrupting both microfilaments and microtubules with inhibitors, we illustrate how each part of the cytoskeleton directs organelle positioning and motility into and within the tip.

Materials and methods

Pollen grains of *Picea abies* (L.) Karst. were field-collected near Stockholm, Sweden, stored at −20°C, and remain viable for years. These grains were sown directly in liquid medium (500 mM sucrose, 1 mM $CaCl_2$, 1 mM H_3BO_3, in distilled H_2O) and grown for 24 h at 30°C. A 25-μl drop of medium containing the pollen tubes was placed on a warm coverslip slide containing 25 μl of 1.5% low-melt agarose (Sigma Type VII) in culture medium. The slide was put at 4°C for 10 s, 200 μl of medium was then added, and the slide put in a humid chamber at 25°C for 1 h so elongation could resume. Stock solutions of amiprophosmethyl (APM), propyzamide, or oryzalin, which disrupt microtubules (Hoffman and Vaughn 1994) in conifer pollen tubes (Anderhag et al. 2000) or latrunculin B (LATB), which disrupts microfilaments in angiosperm pollen tubes (Gibbon et al. 1999) were prepared in 100% ethanol. Stocks were serially diluted into liquid culture medium containing fluorescein isothiocyanate (FITC)–dextran (4.4 kDa). This lowers the ethanol concentration to 1%, which has no effect on *P. abies* pollen tube elongation (Anderhag et al. 2000). A large-tip injection needle (diameter about 70 μm) was back-loaded with 5 μl of inhibitors in culture medium with FITC–dextran. Since *P. abies* tubes slowly elongate at 20 μm h^{-1} in culture (Anderhag et al. 2000), time-lapse images over 10 min [600 frames at 1 frame per second (fps)] were captured to ensure tips were actively elongating. Images were captured with a CCD camera [Micromax 1300-Y (Roper Scientific), using IP Lab software (Scanalytics)] through a 1.3 NA 40× oil lens on a Nikon TE-300 inverted microscope equipped with Nomarski optics. Once an elongating tube was identified, the needle tip was manipulated about 600 μm away from tube. In control applications of liquid medium, FITC–dextran and 1% ethanol, this distance allowed the slight pressure wave from injection into the agar matrix to dissipate so there was no effect on elongation or cytoplasmic streaming within the pollen tube. The full 5 μl of inhibitor was then loaded into the agar medium. Stock solutions were made so the final effective inhibitor concentrations result from complete diffusion of the 5 μl throughout the 50 μl of agar medium and pollen tubes. These effective concentrations were 50 μM for APM, propyzamide and oryzalin (Anderhag et al. 2000) and 5 nM for LATB (Gibbon et al. 1999). The FITC–dextrans were detected by epifluorescence to illustrate that the applied solution passes across the pollen tube tip. Subsequent time-lapse sequences (10 min at 1 fps) illustrate the effects of cytoskeletal disruption on elongation, subcellular morphology, and motility. Sample sizes were 100 pollen tubes for controls, 5 tubes for LATB, 9 tubes for APM, 4 tubes for propyzamide, and 8 tubes for oryzalin.

The endoplasmic reticulum (ER) was labeled with ER tracker Blue White DPX dye (Molecular Probes) following a method in Cole et al. (2000). The dye was diluted to 10 μM (1% dimethyl sulfoxide) into microfuge tubes of liquid culture medium and germinated pollen. Pollen tubes were treated for 30 min in the dark followed by a rinse in culture medium to remove unbound dye. To quantify the fluorescence of the ER tracker dye within the ER, pollen tubes were visualized with epifluorescence microscopy at 20× using a 340- to 380-nm band-pass excitation filter and a 420-nm long-pass emission filter. The fluorescence intensity was captured with the Micromax 1300-Y camera with 100-ms exposures. Initial fluorescence images were captured and then pollen tubes were treated with control solution or 5 nM LATB as above; subsequently images were captured every 10 min for 1 h. To quantify ER fluorescence, pixel grey values were identified within a wide rectangular region extending from the amyloplast zone to the tube apex. To compare intensity over time and between pollen tubes, grey values among the amyloplasts were equalized to zero at the upper edge of the measurement region.

To examine the effect of microtubule disruption on microfilament organization, pollen tubes were grown for 24 h in medium containing 100 μM APM, 100 μM propyzamide, or 100 μM oryzalin. Tubes were then chemically fixed, freeze-shattered, immuno-

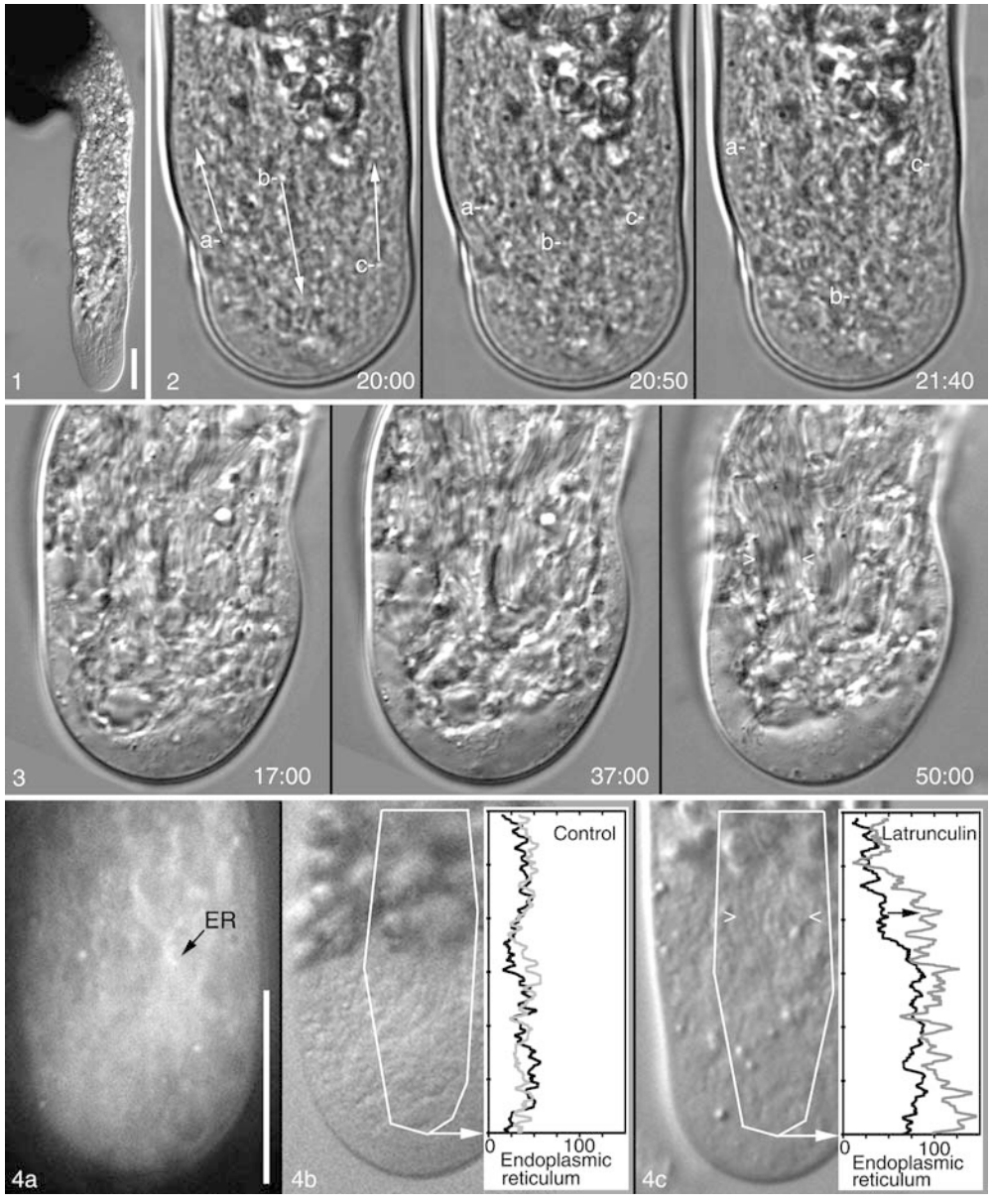
labeled with a monoclonal antibody to pea root actin (Andersland et al. 1994) and a Cy3-conjugated secondary antibody, and examined by confocal microscopy as described in Anderhag et al. (2000).

Results

There are two distinct zones within elongating conifer pollen tubes (Fig. 1). One zone begins in the grain and extends towards the tip, containing the tube nucleus, amyloplasts, vacuoles, and other organelles. The second is at the tip, where amyloplasts are excluded in a clear zone of vesicles, mitochondria, ribosomes, and the endomembrane system. In *Picea abies*, this zone extends back about 30 μm from the tip to the edge of the amyloplasts. There is no inverted cone of secretory vesicles in the tip as seen in angiosperm pollen tubes.

Organelles in conifer pollen tubes stream in a fountain pattern into the tip. In *P. abies*, organelles move consistently in a fountain pattern or rarely in a random pattern or a reverse fountain at the elongating tip. Time-lapse video captured at 1 fps provides a clear image of vesicle and organelle movement within the elongating tip (Fig. 2). Small organelles, including mitochondria and vesicles, move slowly within the center of the tube along clearly defined paths into the 30-μm-long clear zone at the tip. Once in the tip, these organelles move randomly and eventually migrate out of the tip along defined paths in the cell cortex beneath the plasma membrane.

The fountain streaming pattern is dependent on intact actin microfilaments. The application of 5 nM LATB outside the growing pollen tube inhibits elongation, and the fountain pattern of streaming completely stops (Fig. 3). Organelle motility is reduced to only



Figs. 1–4 Elongating pollen tubes of *Picea abies*. Bars = 25 μm (Figs. 2, 3 and 4 are at the same magnification). Video sequences of Figs. 2 and 3 are available as Electronic Supplementary Material

Fig. 1 Pollen tubes have a plastid-free clear zone at the tip. This clear zone lacks an inverted cone of vesicles

Fig. 2 Organelles move in a fountain pattern into the tip. This sequence was captured 20 min (20:00) after application of a control solution of FITC–dextran in germination medium with 1% ethanol. The vesicle labeled *b* moves towards the tip in the tube center while vesicles *a* and *c* move away from the tip along the cell cortex

Fig. 3 Latrunculin B stops growth and completely stops streaming; only Brownian motion is observed. Thin membrane tubules (within *darts* at 50:00) steadily accumulate (17:00–50:00 min after application) in the tip but are not moving

Fig. 4 **a** Endoplasmic reticulum is uniformly distributed from among the amyloplasts and into the clear zone. **b** In controls, this initial distribution (*black plot*) is unchanged after 50 min (*grey plot*). **c** Fifty minutes after application of LATB (*grey plot*), ER accumulates above initial levels (*black plot*), preferentially in the clear zone. The membrane tubules that accumulate following LATB application (within *darts*) are ER (*arrow* in graph). Units for ER (**b, c**) are dimensionless pixel grey values

Brownian motion. However, the clear zone of organelles persists in the extreme tip, even after 15 h. The disruption of microfilaments also causes the accumulation of thin membrane tubules throughout the tip cytoplasm (Fig. 3). These tubules steadily accumulate over at least 15 h after microfilament disruption and are ER. In control pollen tubes, ER is distributed from among the amyloplasts into the clear zone (Fig. 4a) but the relative distribution remains unchanged over time (Fig. 4b). However, following LATB application, the staining intensity of ER quantitatively increases over time within the clear zone (Fig. 4c) coincident with the accumulation of the membrane tubules (Fig. 3).

The fountain streaming pattern and tip organization are also dependent on intact microtubules. The disruption of these microtubules with APM, oryzalin or propyzamide alters streaming and organization in distinct ways and results in reorganization of the actin microfilaments. The application of 50 μM APM stops growth and disrupts the fountain streaming pattern (Fig. 5). Streaming is not completely stopped but is reduced to organelle movement along short, randomly oriented distances. The organization of vesicles and organelles in the extreme tip collapses following microtubule disruption with APM, leaving cytoplasm extending back from an intact plasma membrane (Fig. 5). Actin microfilaments are also disrupted as the organization of vesicles in the tip collapses following APM application (Fig. 6).

Microtubule disruption from propyzamide or oryzalin causes the *reversal* of streaming as elongation stops and causes the reorganization of actin microfilaments. Following the application of 50 μM propyzamide, large membrane tubules migrate into the tip (Fig. 7). These tubules are structurally distinct from the thinner tubules that collect following LATB-induced microfilament disruption. Between 5 and10 min after exposure to propyzamide, the fountain streaming pattern stops, is briefly random, and then these tubules begin to stream in an accelerating reverse fountain (Fig. 7). Actin microfilaments also reorganize from a diffuse pattern throughout the tip (Fig. 8a) into distinct bundles outlining the reverse fountain pattern (Fig. 8b). The application of 50 μM oryzalin also causes the reversal of streaming but the subcellular organization within the tip differs from that of propyzamide-treated cells. Following oryzalin application, large vacuoles, instead of tubules, migrate into the tip in a reverse fountain pattern (Fig. 9). Microfilaments are also reorganized into distinct bundles in the cytoplasm between the migrating vacuoles (Fig. 10).

We conclude that microtubules control the positioning of organelles into and within the tip and influence the direction of streaming by mediating microfilament organization.

Discussion

Our working model is that microfilaments and microtubules coordinate to drive organelle motility and tip extension. Microfilaments and microtubules are co-aligned in a longitudinal array that extends from the amyloplasts into the base of the clear zone (Lazzaro 1996, 1999; Anderhag et al. 2000) and this is the specific region where the fountain pattern of streaming is observed. The disruption of microtubules alters tip organization, including microfilament organization, and causes the reversal of streaming while microfilament disruption completely stops streaming.

Why are there unique disruption patterns following each microtubule inhibitor? Streaming is reduced to short-distance movement in random directions following APM application and this may be due to the specific disruption of the longitudinal microtubule array extending from the amyloplasts into the clear zone (Lazzaro 1999) coincident with the observed disruption of microfilaments. APM application also causes the collapse of cytoplasmic organization in the tip, but leaves the plasma membrane intact. When pollen grains are germinated in medium with APM, a significant subset of pollen tubes has swollen tips where microtubules are specifically disrupted (Anderhag et al. 2000). It is likely that the introduction of APM around elongating tips causes the disruption of both the radial array of microtubules that coordinates cell wall formation in the tip (Lazzaro et al. 2003) and the longitudinal microtubule array that organizes microfilaments.

The results of microtubule disruption following oryzalin and propyzamide are different from the APM applications. Here, streaming switches from a fountain to a reverse fountain pattern as tip organization remains intact but actin microfilaments reorganize into distinct bundles. For propyzamide, this result is consistent with our previous work where pollen tubes growing in medium with propyzamide are shorter and have fragmented

►

Figs. 5–10 Microtubule disruption alters motility and microfilament organization. Bar = 25 μm (for all figures). Video sequences of Figs. 5, 7 and 9 are available as Electronic Supplementary Material

Fig. 5 Amiprophosmethyl stops growth as the fountain streaming pattern is disrupted with organelles moving randomly over short distances. The organization of vesicles and organelles in the extreme tip collapses (10:00–26:40 min after application), leaving cytoplasm extending back from the tip and an intact plasma membrane

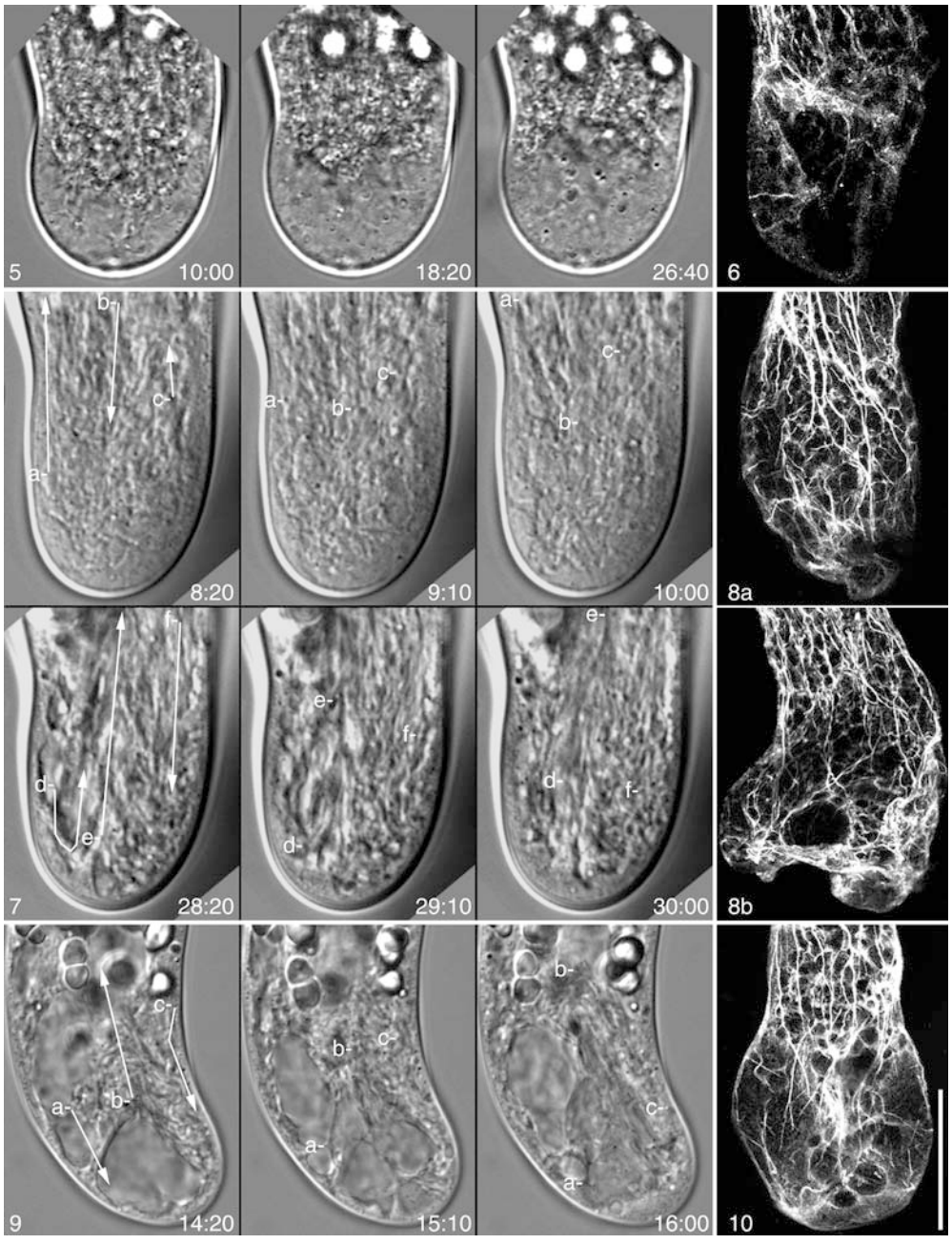
Fig. 6 Microfilaments are disrupted as the extreme tip collapses

Fig. 7 Before application of propyzamide, organelles (*a–c*) stream in a fountain pattern (8:20–10:00) coincident with microfilament bundles. Propyzamide (applied at 10:00 min) stops elongation and causes the reversal of organelle streaming (28:20–30:00) as tubules and thin vacuoles (*d–f*) move towards the tip along the cell cortex and away from the tip down the tube center

Fig. 8a, b Before application of propyzamide, microfilament bundles project throughout the clear zone but are depleted at the extreme tip (**a**). After propyzamide application, microfilaments reorganize into discrete bundles surrounded by microfilament-depleted regions of cytoplasm (**b**)

Fig. 9 Oryzalin also stops elongation and causes large vacuoles to move into the tip (14:20–16:00 min after application) in a reverse fountain pattern

Fig. 10 Oryzalin causes increased bundling of microfilaments, coincident with inhibition of elongation and redistribution of vacuoles



microtubules throughout the tube and into the tip (Anderhag et al. 2000). The application of propyzamide may fragment the longitudinal microtubules throughout the clear zone thus altering streaming patterns by inducing microfilament reorganization.

Organelle motility also switches direction following oryzalin application, although different organelles move within the clear zone than those moving following propyzamide treatment. The vacuolar components of the endomembrane system that are normally constrained amongst the amyloplasts migrate into the clear zone and stream in a reverse fountain pattern as microfilaments reorganize into distinct bundles.

Although microtubule disruption alters streaming patterns, only microfilament disruption by LATB completely stops streaming. This suggests that the microfilament–myosin system is the underlying mechanism for motility, similar to flowering-plant pollen tubes (Hepler et al. 2001). However, there is an unusual change in cell structure following microfilament disruption. Thin tubules of ER rapidly accumulate in the tip and continue accumulating over long time periods (observed up to 15 h). Pollen tubes remain alive during this time but do not elongate. These thin tubules are probably ER. They stain with an ER-specific fluorescent dye and the staining intensity increases within the clear zone over time following LATB application. Our current model is that microfilament-based motility drives vesicle transport from the ER to the Golgi and when this is disrupted, membrane synthesis continues on the ER. These tubules

are coincident with the longitudinal microfilaments and microtubules seen in living pollen tubes (Lazzaro 1999; Anderhag et al. 2000). In other plant cells, intact microfilaments but not microtubules are required for transport between the ER and Golgi and for movement of the Golgi bodies (Brandizzi et al. 2002).

When microfilaments are disrupted by LATB, fountain streaming completely stops and thin tubules rapidly accumulate in the tip. When microtubules are disrupted, streaming is either disrupted or *reverses direction* to a reverse fountain. One possibility is that an underlying reverse fountain pattern directed by myosin along microfilaments is normally masked by the fountain pattern controlled by microtubule-based motility. Two new microtubule motor proteins have been recently isolated from *Nicotiana tabacum* pollen tubes and they are functionally related to kinesins (Cai et al. 2000; Romagnoli et al. 2003). Once microtubules are disrupted with propyzamide or oryzalin, motility would then switch to myosin-based movement along microfilaments. The major flaw in this idea is that motility completely stopped following microfilament disruption with LATB.

An alternative and perhaps more likely scenario is that microtubules function as organizing elements, positioning microfilaments so that myosin-based motility generates a fountain pattern. The disruption of microtubules might cause the reorganization of microfilaments into a reverse fountain pattern. We find that microtubule disruption does change the organization of microfilaments coincident with the observed changes in streaming. These results suggest that microtubules have a direct role in positioning the microfilaments, which fits with the evidence that microtubules act as guide elements for microfilaments in root hairs of *Hydrocharis dubia* (Tominanga et al. 1997), where organelles normally move in a reverse fountain pattern. This movement is reversibly inhibited by microfilament disruption by cytochalasin B but not affected by microtubule disruption by propyzamide. However if both microfilaments and microtubules are disrupted, streaming will not recover until both cytochalasin B and propyzamide are removed from the root hair (Tominanga et al. 1997). A protein that interacts with both microtubules and microfilaments would likely be required for microtubules to control microfilament organization. The kinesins with calmodulin-binding properties (KCBPs) also have myosin sequence homology in their tail regions (Reddy 2001) and might be involved in this interaction. Mutations in the ZWICHEL gene, which encodes a KCBP in *Arabidopsis*, inhibit pollen tube growth (Krishnakumar and Oppenheimer 1999). Recently, a novel kinesin that binds actin microfilaments at the tail domain and binds microtubules in an ATP-dependent mechanism at the head domain has been identified in *Dictyostelium discoideum* (Iwai et al. 2003). This kinesin, DdKin5, bundles microfilaments in vitro and is enriched within actin-rich surface protrusions in the cell.

In conclusion, microtubules within the elongating tip influence microfilament organization and, by doing so, control the direction of organelle streaming into the tip. The underlying organizational role of microtubules in controlling microfilament-dependent motility and the direction of organelle movement has not been observed in flowering-plant pollen tubes and appears unique to conifer pollen tubes. This alternative model system continues to provide new information about the mechanisms underlying polarized cell growth.

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