

The actin microfilament network within elongating pollen tubes of the gymnosperm *Picea abies* (Norway spruce)

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Summary. Actin microfilaments form a dense network within pollen tubes of the gymnosperm Norway spruce (*Picea abies*). Microfilaments emanate from within the pollen grain and form long, branching arrays passing through the aperture and down the length of the pollen tube to the tip. Pollen tubes are densely packed with large amyloplasts, which are surrounded by branching microfilament bundles. The vegetative nucleus is suspended within the elongating pollen tube within a complex array of microfilaments oriented both parallel to and perpendicular with the growing axis. Microfilament bundles branch out along the nuclear surface, and some filaments terminate on or emanate from the surface. Microfilaments in the pollen tube tip form a 6 µm thick, dense, uniform layer beneath the plasma membrane. This layer ensheathes an actin depleted core which contains cytoplasm and organelles, including small amyloplasts, and extends back 36 µm from the tip. Behind the core region, the distinct actin layer is absent as microfilaments are present throughout the pollen tube. Organelle zonation is not always maintained in these conifer pollen tubes. Large amyloplasts will fill the pollen tube up to the growing tip, while the distinct layer of microfilaments and cytoplasm beneath the plasma membrane is maintained. The distinctive microfilament arrangement in the pollen tube tips of this conifer is similar to that seen in tip growth in fungi, ferns and mosses, but has not been reported previously in seed plants.

Keywords: Actin; Confocal microscopy; Cytoskeleton; Gymnosperm; Microfilaments; *Picea abies*; Pollen tubes.

Introduction

Actin microfilaments are crucial for tip growth in pollen tubes (reviewed in Steer 1990, Tiezzi 1991, Pierson and Cresti 1992, Derksen et al. 1995b). Microfilaments are enriched at the tip (Perdue and Parthasara-

thy 1985, Pierson et al. 1986, Pierson 1988, Tiwari and Polito 1988, Tang et al. 1989b, Heslop-Harrison and Heslop-Harrison 1991), and disruption of the actin cytoskeleton with inhibitors blocks tip growth (Lancelle and Hepler 1988, Heslop-Harrison and Heslop-Harrison 1989a, Heslop-Harrison et al. 1991). Myosin and actin are both present at the extreme tip of *Nicotiana* pollen tubes, forming a cap-like structure (Pierson 1988, Tirlapur et al. 1995). The actin-myosin system functions to deliver vesicles to the growing pollen tube tip (reviewed in Pierson and Cresti 1992), but microfilaments may also shape the pollen tube by resisting the osmotic forces driving tip expansion (proposed in Picton and Steer 1982).

The actin-myosin system has recently been identified in gymnosperm pollen tubes. Microfilaments are reported to be absent from pollen tube tips in *Pinus densiflora* (Terasaka and Niitsu 1994), but are present throughout the pollen tube, including the tip, in *Pinus sylvestris* (de Win et al. 1996). To date, the cytoskeleton has only been investigated within pollen tubes in the genus *Pinus*. In *Picea abies* (Norway spruce, Pinaceae), pollen grains land in the pollination droplets on female cones in the spring and germinate within a cleft in the micropyle (Singh 1978). During the subsequent 3–4 weeks, pollen tubes grow 100–200 µm through the integument and neck cells to reach the archegonium and fertilize the egg cell (Christiansen 1972, Singh 1978). In Pinaceae, the integument cells degenerate in the path of the elongating pollen tube (Owens and Morris 1990, Dawkins and Owens 1993) as the tip secretes hydrolase, acid phosphatase, este-

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rase, amylase, and protease (Pettitt 1985). Integument cells which come in contact with the sides of the elongating pollen tube are mishapen and pushed aside but not degraded (Owens and Morris 1990).

In this report, detailed microscopic analysis of the actin microfilament network in *P. abies* is presented using confocal microscopy and three-dimensional image analysis of optical serial sections. Of particular interest is the finding that a distinct microfilament layer forms beneath the plasma membrane of the pollen tube tip, ensheathing an actin depleted core. This structure is similar to the microfilament cap in fungal hyphae tips (reviewed in Heath 1994) and the actin collar in fern and moss protonemal tips (Kadota and Wada 1992, 1995; Meske and Hartmann 1995), but has not been reported in gymnosperm or angiosperm pollen tubes. In *P. abies*, it may function to add strength and stability to the wide pollen tube tips as it slowly elongates through a milieu of secreted hydrolytic enzymes.

Material and methods

Male cones from *Picea abies* (L.) Karch were collected from a seed orchard 10 km from Stockholm, Sweden and kept in the laboratory at 20 °C for 10 days while the scales opened to shed pollen which was

then stored at -20 °C. Pollen grains were taken directly from -20 °C and scattered on a layer of 1% agar containing 10% sucrose, 1 mM calcium chloride, and 1 mM boric acid (Pettitt 1985). After 24 h in a humid chamber at 30 °C (Frankis and Grayson 1990), 95% of the pollen grains had germinated, with pollen tubes 100–150 µm long. The vegetative nucleus was typically in the center of the elongating tube. Elongating tubes were fixed and permeabilized for 1 min with 66 mM PIPES (pH 7.0) containing 0.1% Triton X-100 and 0.3% paraformaldehyde. This buffer was pipetted directly onto pollen tubes growing along the agar surface to avoid disturbing the microfilaments. After 1 min, pollen tubes were gently transferred to microfuge tubes using a glass Pasteur pipette. Stock solutions of rhodamine phalloidin (in methanol) and propidium iodide (in distilled water) were then added to a final concentration of 0.72 µM rhodamine phalloidin and 1.67 µg/ml propidium iodide in a final volume of 200 µl. Pollen tubes were stained for 5 min as they settled towards the base of the microfuge tube under the force of gravity. The majority of the staining solution was then removed and replaced with PIPES buffer containing TX-100 and paraformaldehyde to a final volume of 500 µl. Pollen tubes were then examined immediately. The entire sample was discarded after 10 min, since initial observations showed that the microfilament network began to deteriorate after 20 min. To measure autofluorescence, controls were prepared using identical experimental methods, except that rhodamine phalloidin and propidium iodide were omitted. In addition, identical confocal settings were used for both treated pollen tubes and controls. Pollen tubes were examined with a Bio-Rad MRC 600 laser scanning confocal microscope, using the rhodamine filter set (10 nm wide exciting laser line at 488 nm and a 515 nm barrier emission filter), and the ×60 oil immersion objective (Nikon). Nomarski DIC imag-

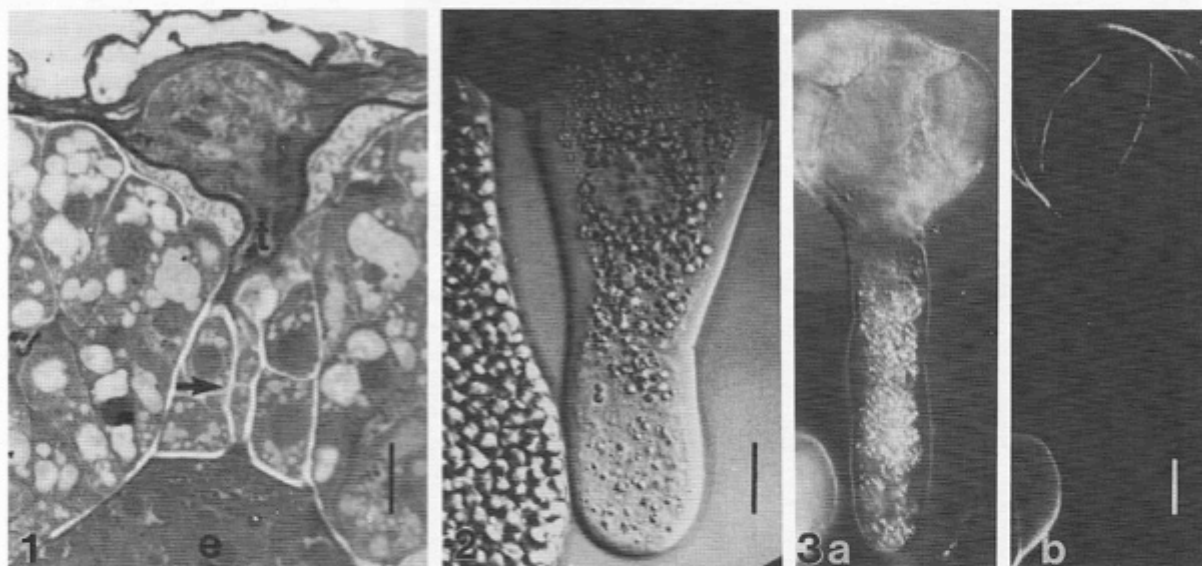


Fig. 1. A pollen grain of Norway spruce germinates in vivo within the micropylar cleft on the megagametophyte, and the tube (*t*) will grow through the neck cells (arrow) to reach the egg cell (*e*). This pollen tube is 20 µm wide, and will grow 100 µm to reach the egg cell

Fig. 2. As pollen tubes elongate from the grain, they fill with amyloplasts. The microfilament network of this pollen tube is shown in Fig. 4. In addition, the tube to the left is completely filled with large amyloplasts

Fig. 3. In unstained control pollen tubes, the only autofluorescence observed through the confocal microscope was from the pollen grain walls. Nomarski DIC (*a*) and confocal fluorescence (*b*) pair. Bars: 20 µm

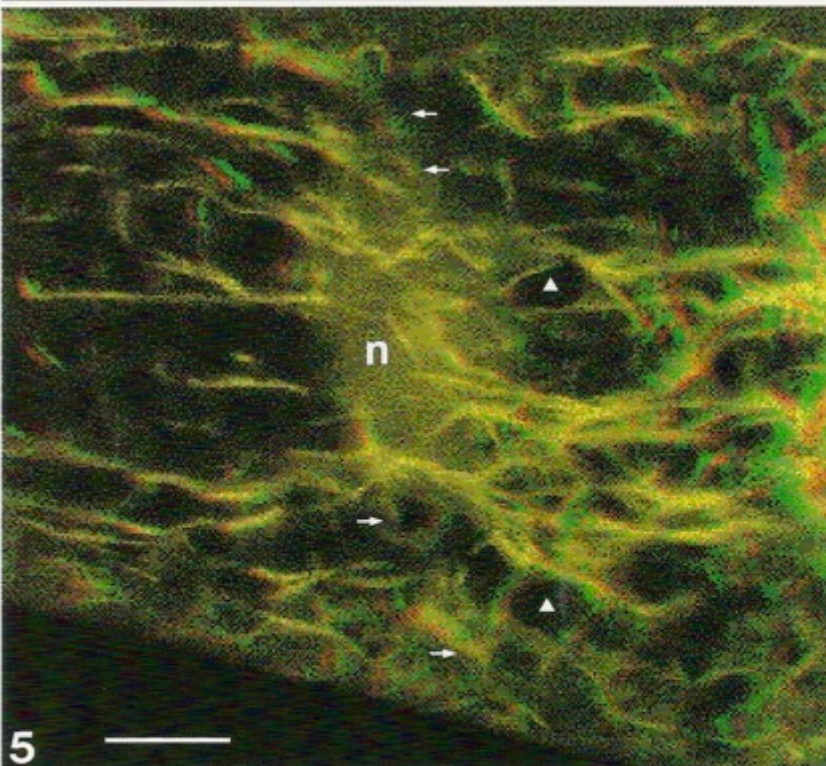
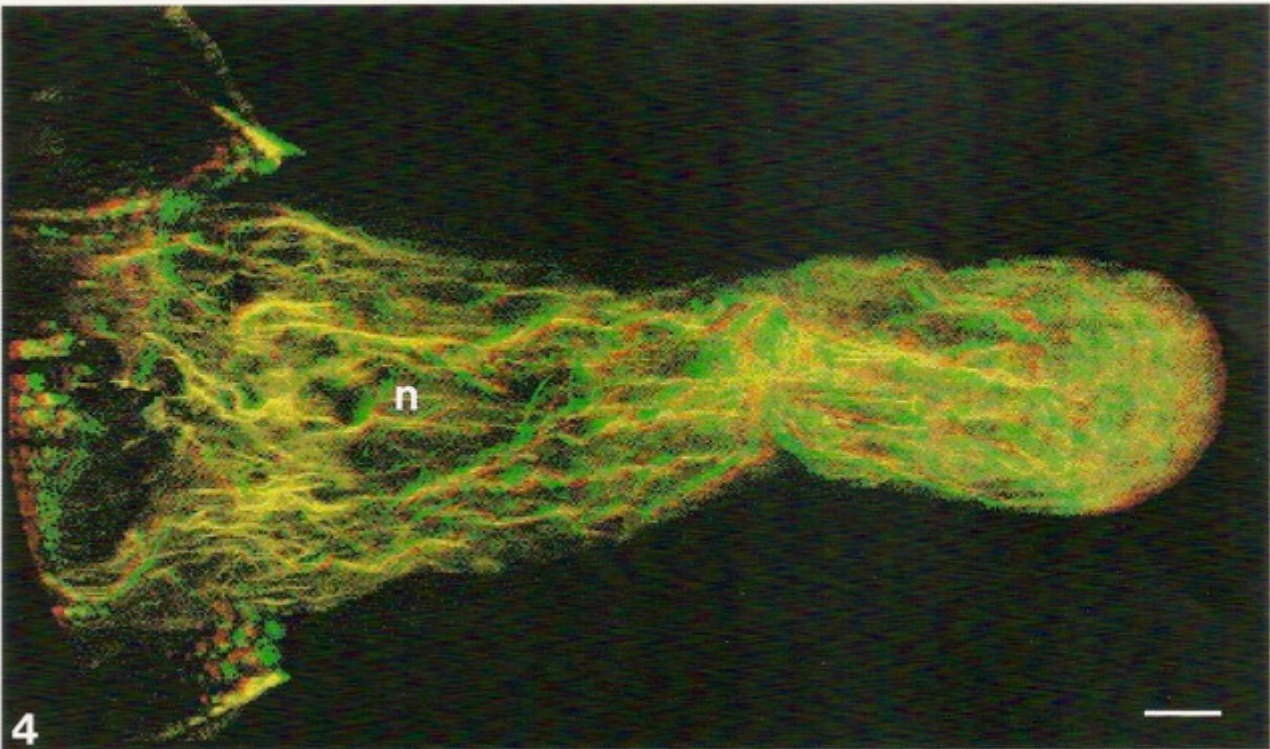


Fig. 4. Actin microfilaments emanate from within the pollen grain and extend down the length of the pollen tube in this red-green stereo pair. They twist and branch in three dimensions, forming microfilament bundles of varying thickness. The vegetative nucleus (*n*) is in the center of the elongating tube. The filament network is more dense at the growing tip. This is a stack of 16 optical sections projected as a 15° stereo pair and is best viewed with red-green stereo glasses. With the red filter over the left eye, the viewer is looking at the pollen tube from the outside. However, if the red filter is placed over the right eye, the viewer is inside the pollen tube looking out, and can see the microfilament layer and actin depleted core at the pollen tube tip

Fig. 5. The vegetative nucleus (*n*) is suspended within the microfilament network. Filament bundles extend towards and branch along the nuclear surface, primarily in the direction of pollen tube elongation (towards the right in this micrograph). In addition, filaments oriented perpendicular to the growing axis project from the nucleus towards the plasma membrane (arrows). Individual amyloplasts (arrowheads) are also suspended within the microfilament network, as filament bundles branch and twist around them. This is a stack of 20 optical sections projected as a 10° stereo pair. Bars: 10 μ m

es were collected simultaneously through the confocal scan head with a fiber optic cable connected through the second filter block. Confocal images were enhanced with Kalman averaging for 10 scans. Optical serial sections were collected at 1 μm intervals at a thickness of 0.5 μm . Subsequent generation of red-green stereo pairs, three-dimensional rotation of pollen tube tips, and quantitative measurements were completed with "NIH Image" software on a Macintosh computer. Median optical sections were used for the measurement of pollen tube tips, and the measurements were calibrated with an image of a stage micrometer recorded through the confocal microscope.

The micrograph in Fig. 1 was taken from a toluidine blue stained 900 nm thick section of a *P. abies* ovule dissected from a female cone, fixed in 3.0% paraformaldehyde and 1.25% glutaraldehyde in 50 mM phosphate buffer (pH 7.0), and embedded in LR White resin.

Results

Pollen grains of *Picea abies* germinate *in vivo* within the micropylar cleft adjacent to the archegonium, and the pollen tube grows out of the grain through several neck cells towards the egg cell (Fig. 1). The tubes are densely filled with amyloplasts as they elongate (Fig. 2). In the confocal microscope, the only autofluorescence observed in unstained controls was from the pollen grain walls (Fig. 3 a, b). Pollen tubes germinated *in vitro* (Figs. 2 and 3) were similar in length and width to tubes *in vivo* (Fig. 1), and were subsequently labeled with rhodamine phalloidin and propidium iodide to visualize the microfilament cytoskeleton and vegetative nucleus. Actin microfilaments emanate from within the pollen grain and form a long network parallel to the direction of tube elongation (Fig. 4). The network contains microfilament bundles of varying thickness, and is more dense at the growing tip (Fig. 4). The vegetative nucleus is suspended within this network of actin filaments in three dimensions, as filaments twist towards and away from the nucleus (Fig. 5). The majority of microfila-

ments connected to the nucleus are parallel to the direction of pollen tube growth. However, microfilaments perpendicular to the growing axis also connect to the nuclear surface and radiate towards the pollen

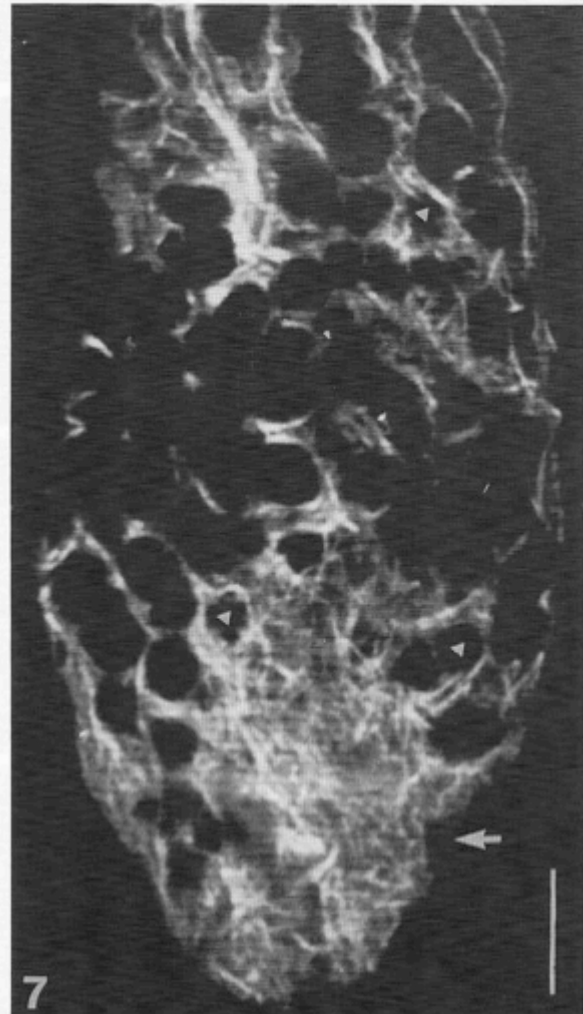
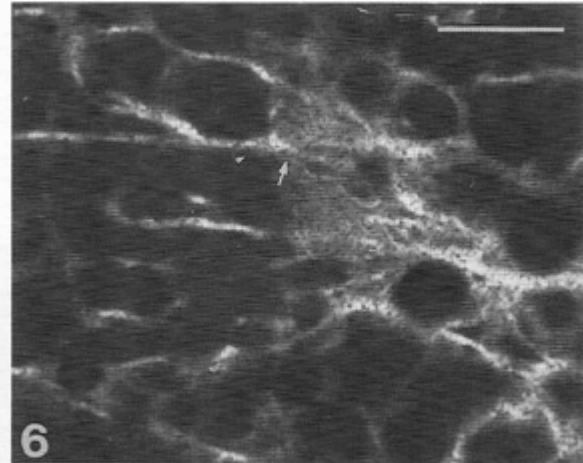


Fig. 6. Microfilaments run along the nuclear surface in this optical section of the edge of the vegetative nucleus in Fig. 5. From this 0.5 μm thick section, it is clear that microfilament bundles branch along the nuclear surface (arrow). Some filaments appear to terminate on the nuclear surface, while others are bent where they contact the nucleus. This micrograph is oriented so the pollen tube tip is to the right

Fig. 7. Amyloplasts (three labeled with arrowheads) are enmeshed by microfilaments which bend around them in this single optical section (0.5 μm thick). In addition, the actin filament network is dense at the pollen tube tip (arrow), where the section passes through the layer of microfilaments beneath the plasma membrane. This pollen tube was not treated with paraformaldehyde. Bars: 10 μm

tube plasma membrane (Fig. 5). Amyloplasts, which appear as black ellipsoids in the confocal, are also suspended within the actin network (Fig. 5). In a single optical section (0.5 μm thick) along the surface of the vegetative nucleus in Fig. 5, microfilament bundles lead up to and branch along the surface of the nucleus, and microfilaments appear to terminate on the surface or bend along the nuclear envelope (Fig. 6). In addition, from single optical sections it is clear that microfilament bundles branch and twist around individual amyloplasts (Fig. 7).

A distinctive distribution of microfilaments occurs at the growing tip of *P. abies* pollen tubes. In glancing optical sections, the microfilament network appears to extend across the tube tip (Figs. 7 and 8 a). However, median optical sections reveal that a layer of actin filaments is present beneath the plasma membrane at the growing tip and an actin depleted core is formed in the tube tip, ensheathed by this microfilament layer (Fig. 8 b). Microfilaments are present throughout the pollen tube behind this core region, and appear to terminate at the edge of the core, or occasionally contin-

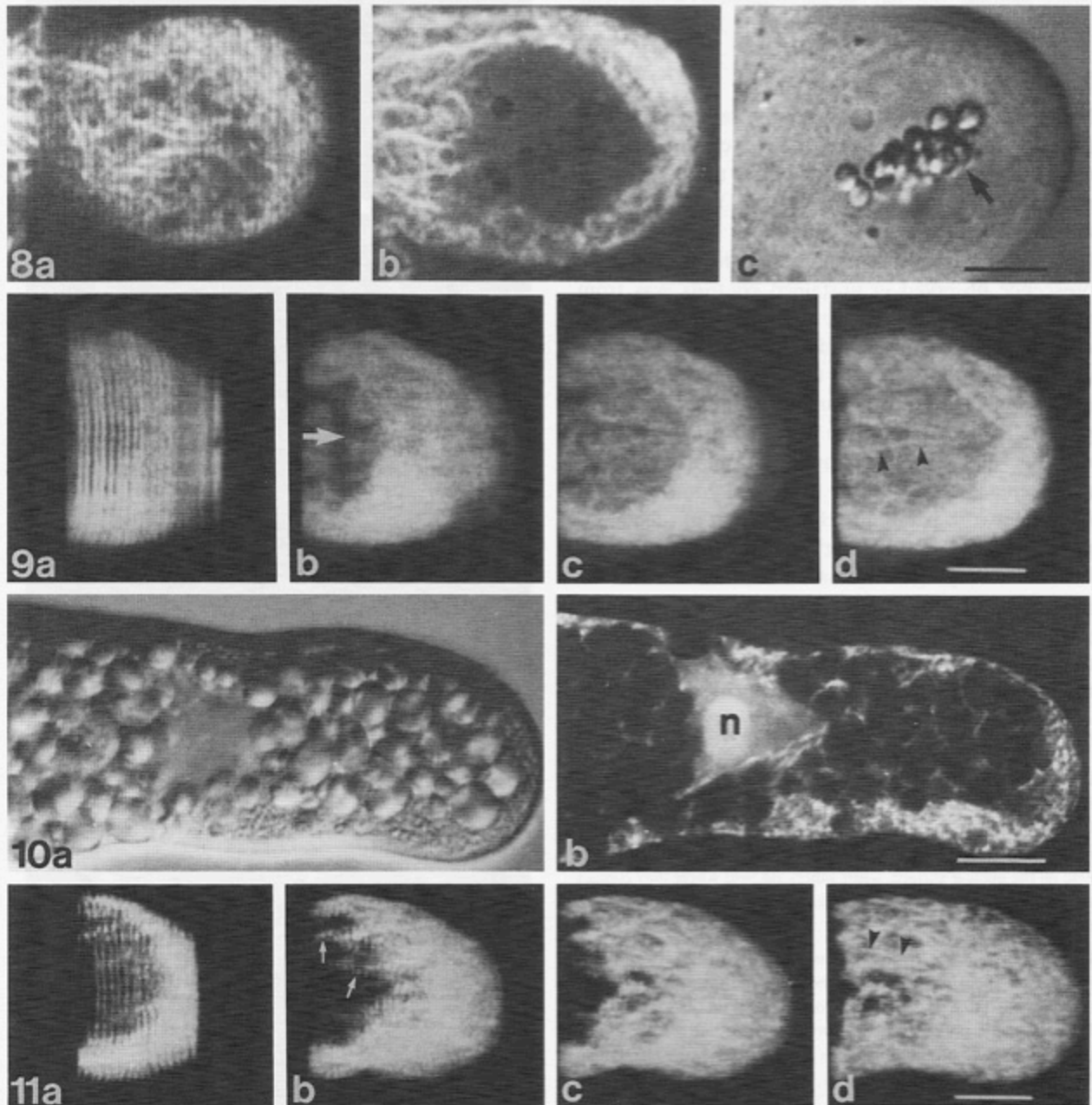


Table 1. Dimensions of the layer of actin microfilaments beneath the plasma membrane and of the actin depleted core in median optical sections through the tips of elongating pollen tubes

Microfilament band at tip, thickness	6.13 ± 0.41 μm
Actin depleted core	
width	15.14 ± 1.47 μm
length	30.21 ± 2.93 μm

Width of the filament layer was measured at equally spaced points around the tube tip. Core width was measured at the core center, and core length was measured along the central axis of elongation; $n = 36$ for filament layer thickness and 9 for core width and length. Data are means ± standard error. The microfilament layer beneath the plasma membrane, and the actin depleted core, extend back 36.34 μm from the pollen tube tip

ue into it (Fig. 8 b). This core is not a vacuole, it contains cytoplasm and small amyloplasts (Fig. 8 c). When optical serial sections of the pollen tube tip are stacked and rotated in three dimensions, it is clear that this microfilament layer is uniform around the elongating tip and ensheathes the actin depleted core with in the center of the pollen tube tip (Fig. 9). The average thickness of the layer of microfilaments surrounding the actin depleted core was 6.13 μm (Table 1). The actin depleted core was 15.14 μm wide, and 30.21 μm long. Both the microfilament layer and actin depleted core extended back 36.34 μm from the pollen tube tip (Table 1).

The majority of large plastids are usually excluded from the actin depleted core (Fig. 8 c), but this exclusion is not absolute in *P. abies*. Large amyloplasts will migrate into this actin depleted core region, filling the pollen tube up to the growing tip (Fig. 10 a). How-

ever, the distinct layer of microfilaments remains beneath the plasma membrane, coincident with the remaining layer of cytoplasm and small organelles (Fig. 10 a, b). The microfilament layer remains uniform when viewed in three-dimensional reconstruction (Fig. 11 a, b). In addition, microfilament bundles are clustered around the large amyloplasts that have migrated into the actin depleted core (Fig. 11 b). Within the microfilament layer at the pollen tube tip, distinct microfilament bundles are present along the inner surface (Fig. 9 d) near the actin depleted core and along the outer surface (Fig. 11 d) near the plasma membrane.

Discussion

The tip of elongating *Picea abies* pollen tubes contains a 6 μm thick layer of microfilaments which ensheathes an actin depleted core. Peripheral microfilament layers have also been described in tip growth in fungi, ferns, and mosses. A peripheral actin layer is seen in the tips of fungal hyphae (Heath 1987), and changes in this layer predict the direction of tip growth (Jackson and Heath 1993a). In protonemal tips of ferns (Kadota and Wada 1992, 1995), and mosses (Meske and Hartmann 1995), microfilaments within the peripheral band reorient to predict the direction of tip growth during phototropism. In angiosperm pollen tubes, microfilaments form a dense network at the tip, but this is typically a diffuse pattern in epifluorescence microscopy (Perdue and Parthasarathy 1985, Pierson et al. 1986, Tiwari and Polito 1988, Tang et al. 1989b). The fine structure of actin filaments is better preserved in angiosperms with

Fig. 8. Elongating pollen tubes contain a dense network of actin microfilaments at the tip (a). However, median optical sections indicate that these microfilaments form a 6 μm thick layer beneath the plasma membrane, and that an actin depleted central core is present within the tip (b). This central core is not a vacuole, it contains cytoplasm and small amyloplasts (arrow), while the majority of large amyloplasts have not migrated into the pollen tube tip (c)

Fig. 9 a–d. When rotated in three dimensions, it is clear that the microfilament layer beneath the plasma membrane at the tip is uniform and that an actin depleted core is present within the tip. This is a stack of 20 optical sections through the terminal 28 μm of the pollen tube in Fig. 8. In a, the pollen tube is growing out of the printed page to the right at an 85° angle to the page, and continues to turn to the right at 25° intervals in b–d. The interior of the pollen tube and the actin depleted central core come into view in b (arrow), and are more pronounced as the pollen tube tip rotates in space. In d, the viewer is inside the pollen tube tip, within the actin depleted core, looking out towards the microfilament layer: microfilament bundles (arrowheads) running along the interior surface of the microfilament layer are visible

Fig. 10. Large amyloplasts are not always excluded from the elongating tip (a). However, the layer of microfilaments remains beneath the plasma membrane, extending back towards the vegetative nucleus (*n*) (b). Nomarski DIC and confocal pair

Fig. 11 a–d. The peripheral layer of microfilaments beneath the plasma membrane remains uniform after the large amyloplasts have migrated into the actin depleted core. Optical sections of the terminal 31 μm of the pollen tube in Fig. 10 were stacked and rotated in space. In a, the pollen tube is growing into the printed page towards the right at an 85° angle to the page. The pollen tube tip turns to the right at 25° intervals in b–d. Cross sections (arrows) through microfilament bundles adjacent to amyloplasts which have migrated into the tip are seen in b. In d, the viewer is outside the pollen tube tip, looking at the peripheral layer of microfilaments beneath the plasma membrane: microfilament bundles (arrowheads) running along the outer surface of the microfilament layer are visible. Bars: 10 μm

DMSO-microwave fixation, where a microfilament cap is seen at the tip of *Nicotiana* pollen tubes (Pierson 1988), and a fine meshwork of actin filaments is seen through the tips of *Narcissus* pollen tubes (Heslop-Harrison and Heslop-Harrison 1991). However, in *Narcissus* there is no indication of an actin depleted core in epifluorescent images focused at the medial plane of the tube tip (Heslop-Harrison and Heslop-Harrison 1991). Within gymnosperm pollen tubes, actin microfilaments form a long, axial array which extends to the tip in *Pinus sylvestris* (de Win et al. 1996), but is apparently excluded from the tip in *Pinus densiflora* (Terasaka and Niitsu 1994). An actin depleted core within a microfilament sheath has not been reported previously in pollen tube tips.

Conifer pollen tubes secrete a milieu of degradative enzymes in vitro, including hydrolase, protease, acid phosphatase, esterase, and amylase, with unique isozymes produced during tube growth (Pettitt 1985). In *Pseudotsuga* and *Picea*, the integument cells degenerate when in the path of the elongating tip while cells which are not directly in contact with the tip do not degrade, but are merely pushed aside as the tube extends (Owens and Morris 1990, Dawkins and Owens 1993). The microfilament sheath seen in *P. abies* extends back 36 μm from the tip, with microfilaments throughout the rest of the tube in a primarily longitudinal array, so this microfilament sheath may provide added strength to the pollen tube tip as it elongates through degraded cells and degrading enzymes. Picton and Steer (1982) proposed a model for tip growth in which microfilaments shape the tip by providing strength to resist the expansive pressure within the tube, and the microfilament sheath in *P. abies* is evidence in support of this model for gymnosperms. In addition, there is experimental evidence that the peripheral actin band around hyphal tips in the fungus *Saprolegnia* strengthens the tip against osmotic forces, since the tips burst when the band is disrupted with ultraviolet irradiation (Jackson and Heath 1993b).

The microfilament sheath may also function to position organelles within the elongating tip. In pollen tubes from the related conifer *Pinus sylvestris*, mitochondria are aligned in rows beneath the plasma membrane around the tip (de Win et al. 1996), coincident with the microfilament sheath seen in *P. abies*. In addition, quantitative analysis of organelle distribution of pollen tube tips of *Nicotiana tabacum* demonstrates that secretory vesicles are concentrated from 0–6 μm from the tip (Derksen et al. 1995a), coinci-

dent with the 6 μm thick microfilament layer. In pollen tubes from *Pinus sylvestris*, secretory vesicles are also enriched at the tip, but not as dramatically as in *Nicotiana* (de Win et al. 1996).

Inhibitor studies have shown that actin filaments are responsible for tip growth (Lancelle and Hepler 1988, Heslop-Harrison and Heslop-Harrison 1989a, Heslop-Harrison et al. 1991). In *Nicotiana*, myosin is concentrated in a cap at the tip of emerging pollen tubes (Tirlapur et al. 1995), coincident with the microfilament cap seen in DMSO-microwave treated material (Pierson 1988). In conifers, myosin immunolocalization is also concentrated in a layer beneath the tip of *Pinus densiflora* pollen tubes (Terasaka and Niitsu 1994), forming a cap similar in shape to the end of the microfilament sheath seen in *P. abies*. However, in *Pinus densiflora* microfilaments were excluded from the tip region, except when cytoplasmic "granules" migrated into the tip (Terasaka and Niitsu 1994). There is no evidence for a microfilament sheath in *Pinus densiflora*, although microfilaments were labeled with rhodamine phalloidin in 4% formaldehyde for 60 min, followed by a 30 min buffer rinse before examination (Terasaka and Niitsu 1994), so it is difficult to make comparisons between the microfilament organization reported in *Pinus* and that described here for *Picea*. In addition, recent work indicates that actin microfilaments are present in the tip of *Pinus sylvestris* (de Win et al. 1996).

The microfilament sheath is not an artifact. Material was only examined during the first 10 min after treatment with rhodamine phalloidin, since after 20 min the fine structure of the microfilament network began to deteriorate and thick microfilament bundles formed in the tube, including throughout the tip (data not shown). The rhodamine phalloidin solution contained a low concentration of paraformaldehyde (0.3%) and did not contain EGTA. Both components will cause changes in cytoskeletal ultrastructure (Pierson 1988, Heslop-Harrison and Heslop-Harrison 1991, Baskin et al. 1992, He and Wetzstein 1995) and perhaps should not be routinely included at conventional concentrations. However, the inclusion of 0.3% paraformaldehyde made it technically possible to collect optical serial sections. The fine structure of the microfilaments at the tip was also visible in material not treated with paraformaldehyde (Fig. 7), but the cytoskeletal structure visibly deteriorated after 2–3 min.

The vegetative nucleus is enmeshed within a network of microfilaments. Thick filament bundles branch

into thinner bundles around the nucleus, similar to that seen in other plant cells (Seagull et al. 1987, White and Sack 1990). In addition, single microfilament bundles branch out along the vegetative nuclear surface, and in single optical sections, it appears that some of these filaments terminate on the nuclear surface. In angiosperm pollen tubes, inhibitor studies have shown that movement of the vegetative nucleus is driven by microfilaments (Franke et al. 1972, Picton and Steer 1981, Lancelle and Hepler 1988, Heslop-Harrison et al. 1988, Heslop-Harrison and Heslop-Harrison 1989a), and myosin has been localized on the surface of the vegetative nucleus in angiosperm pollen tubes (Heslop-Harrison and Heslop-Harrison 1989b, Tang et al. 1989a, Miller et al. 1995) and in *Pinus densiflora* (Terasaka and Niitsu 1994). Amyloplasts were suspended within a network of microfilament bundles, similar to the relationship between microfilaments and amyloplasts observed in epifluorescence of gravity sensing root tip cells (White and Sack 1990). In addition, distinct microfilament bundles branched around individual plastids within single optical sections of *P. abies* pollen tubes. Myosin antibodies have also been localized on amyloplasts within angiosperm pollen tubes (Heslop-Harrison and Heslop-Harrison 1989b), and are distributed around large, elliptical organelles in *Pinus densiflora* (Terasaka and Niitsu 1994).

In conclusion, microfilaments in pollen tubes of the gymnosperm *P. abies* form a long array which emanates from within the pollen grain and extends the length of the pollen tube, up to and including the tip. Microfilament bundles parallel to the direction of tube growth branch along the surface of the vegetative nuclear envelope, and microfilaments perpendicular to the growing axis radiate from the nucleus towards the plasma membrane. Microfilament bundles also twist and branch around individual amyloplasts. Finally, a distinct 6 μm thick microfilament layer is present beneath the plasma membrane along the terminal 36 μm of elongating pollen tubes, and this layer ensheathes an actin depleted core which contains cytoplasm, organelles, and amyloplasts.

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