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The spermatogenous body cell of the conifer *Picea abies* (Norway spruce) contains actin microfilaments

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Summary. In conifer pollen, the generative cell divides into a sterile stalk cell and a body cell, which subsequently divides to produce two sperm. In *Picea abies* (Norway spruce, Pinaceae) this spermatogenous body cell contains actin microfilaments. Microfilament bundles follow the spherical contour of the body cell within the cell cortex, and also traverse the cytoplasm and enmesh amyloplasts and other organelles. In addition, microfilaments are associated with the surface of the body cell nucleus. The sterile stalk cell also contains microfilament bundles in the cytoplasm, around organelles, and along the nuclear surface. Within the pollen grain, microfilament bundles traverse the vegetative-cell cytoplasm and are enriched in a webbed cage which surrounds the body cell. Microfilaments were identified with rhodamine-phalloidin and with indirect immunofluorescence using a monoclonal antibody to actin. The majority of evidence in literature suggests that the spermatogenous generative cell in angiosperms does not contain actin microfilaments, so the presence of microfilaments within the spermatogenous body cell in *P. abies* appears to be a fundamental difference in sexual reproduction between conifers and angiosperms.

Keywords: Actin; Conifer; Cytoskeleton; Generative cell; Microfilament; *Picea abies*; Pollen.

Introduction

The development of pollen and production of sperm in the conifers is more complex than in the angiosperms, involving several additional mitotic steps. In the angiosperms, the haploid microspore divides mitotically to produce the vegetative cell and the generative cell, and the generative cell subsequently divides to produce the two sperm used in double fertilization. However, in conifers the haploid microspore divides to produce a prothallial cell and a central cell. The central cell divides to produce another prothallial cell and the generative cell. This generative cell then divides to produce the stalk cell and the body cell. Finally, this body cell divides to produce the two sperm, but only one of these has a role in fertilization. When conifer pollen is shed each spring, it is in either a four-cell stage with the two prothallial cells, the vegetative cell, and the generative cell, or in a five-cell stage where the generative cell has divided into the body and stalk cells (for review, see Singh 1978).

Pollen from *Picea abies* is released in the five-cell stage with the body cell and stalk cell. Pollen grains land in the pollination droplets on female cones in the spring, germinate within a cleft in the micropyle, and pollen tubes grow 100–200 μm through the integument and neck cells to reach the archegonium. The body cell and stalk cell then migrate into the tube, and the body cell divides to produce two sperm nuclei (Christiansen 1972). This cell division is incomplete in *Picea* spp., since a partial cell plate forms between the two sperm nuclei in *Picea glauca* (Dawkins and Owens 1993). When the pollen tube penetrates into the egg cell, one sperm nucleus migrates 500 to 1000 μm to the center of the massive cell together with a surrounding layer of plastids, while the other sperm nucleus remains at the top of the egg cell or associates with the ventral canal cell (Singh 1978). Since plastid DNA is paternally inherited in most conifers (Owens and Morris 1990), microfilaments or microtubules may be necessary in the spermatogenous

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body cell so that following mitosis, the sperm nucleus will move through the egg cell with its complement of plastids held intact by the cytoskeleton.

In conifer pollen tubes, the microfilament network has been examined in *P. abies* (Lazzaro 1996) and *Pinus sylvestrus* (de Win et al. 1996) and both microfilaments and microtubules have been examined in *Pinus densiflora* (Terasaka and Niitsu 1994). The arrangement of microfilaments within the pollen grain has only been examined in *Pinus densiflora*, where microtubules, microfilaments, and myosin labeling were identified coincident with the generative cell (Terasaka and Niitsu 1994). However, it is not clear from the evidence presented whether the cytoskeletal elements were actually within the generative cell. They may have been in the surrounding vegetative cell, forming a network around the generative cell. In any case, this generative cell is not the spermatogenous cell in conifers since it will divide to produce the body cell and stalk cell (Singh 1978). The current hypothesis in the angiosperm literature suggests that actin microfilaments are not present in the spermatogenous generative cell (reviewed in Palevitz and Tiezzi 1992), although there is a more recent report of microfilaments in *Lilium longiflorum* generative cells (Knox et al. 1993). The microfilament motor protein myosin was identified within the generative cell of *Nicotiana tabacum* (Tirlapur et al. 1995, 1996), and profilin and possibly g-actin were labeled within generative cells of *Ledebouria socialis* (Hess et al. 1995).

The present study investigates the actin cytoskeletal network within the body cell of *P. abies*. Microfilaments were labeled with phalloidin and a monoclonal actin antibody, and cells were examined with confocal scanning microscopy. Actin microfilaments were identified in the spermatogenous body cell, and this finding may be a fundamental difference between pollen development in conifers and angiosperms, where the majority of evidence indicates that the spermatogenous generative cell lacks microfilaments.

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 in the center of the elongating tube, while the body and stalk cells remained within the grain.

Rhodamine-phalloidin labeling was carried out as described previously (Lazzaro 1996). Pollen was fixed and permeabilized for 1 min with 66 mM PIPES (pH 7.0) containing 0.1% Triton X-100 and 0.3% paraformaldehyde pipetted directly onto pollen tubes growing along the agar surface. After 1 min, pollen tubes were gently transferred to microfuge tubes with 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 of propidium iodide per ml 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. To detect autofluorescence, controls were prepared by identical experimental methods, except that rhodamine-phalloidin and propidium iodide were omitted.

For immunolabeling of actin, germinated pollen was fixed directly on the agar surface for 1 h with 4% paraformaldehyde in PEM buffer (66 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$) at pH 7.0. Fixed pollen was then transferred to microfuge tubes and pelleted by centrifuging at 1400 **g** for 10 s. The pollen was then rinsed three times for 10 min each in PEM buffer at pH 5.0. Pollen tubes in the final pellet were fractured with a plastic pestle which fit in the microfuge tube. This partially ruptured the tube cell wall and increased the enzymatic digestion of the body cell walls and the penetration of antibodies. The body cell wall was digested by agitating for 1 h at 35 °C with 1% cellulase (Onozuka R-10; Serva, Heidelberg, Federal Republic of Germany) and 1% macerase (Macerozyme R-10; Serva) in PEM buffer (pH 5.0) containing protease inhibitors (4 mM phenylmethylsulfonyl fluoride, 20 μg of leupeptin per ml). The resulting fraction was rinsed three times for 5 min each in blocking buffer (phosphate-buffered saline (pH 7.5), 1% BSA, 1% TX-100) (Wick 1993). The pollen sample was then divided into three aliquots for parallel treatment with primary and secondary antibody, secondary antibody alone, or no antibody. The first aliquot was incubated for 24 h at 35 °C in a mouse monoclonal antibody (Amersham N350; Amersham, Buckinghamshire, U.K.) to chicken gizzard actin (diluted 1 : 50 in blocking buffer) which detects plant actin in angiosperm pollen tubes (Tang et al. 1989b, Åström et al. 1991, Sorri et al. 1996). Pollen tubes were then rinsed four times for 10 min each in blocking buffer and then incubated for 1 h at 35 °C with a Cy3-conjugated goat secondary antibody (Sigma C2181; Sigma, St. Louis, MO, U.S.A.) to mouse immunoglobins, diluted 1 : 200 in blocking buffer. The pollen tubes were then rinsed three times for 10 min each in blocking buffer and mounted in Moviol (Hoechst, Frankfurt, Federal Republic of Germany) with n-propyl gallate to retard photobleaching (Wick 1993). As a control, the second aliquot was processed in parallel with the first, but incubated in blocking buffer without primary antibody followed by incubation with the Cy3-conjugated secondary at 1 : 200 to evaluate nonspecific binding of the secondary antibody. The third aliquot was processed in parallel with the first and second, but incubated in blocking buffer without primary or secondary antibodies to measure autofluorescence.

Pollen grains were examined on a Bio-Rad MRC 600 confocal laser scanning microscope with a 568 nm excitation line, a 585 nm long-pass emission filter, and the Nikon ×60 oil immersion objective (Bio-Rad, Hertsfordshire, U.K.). Nomarski DIC images were col-

lected simultaneously through the confocal scan head. Confocal images were enhanced with Kalman averaging for 10 scans. Optical serial sections were collected at a thickness of 0.5 μm. Confocal projections were generated by digitally combining adjacent serial sections into a single image with the public-domain program NIH Image on a Macintosh computer. Identical confocal settings were always used for both treated pollen tubes and controls.

For electron microscopy, germinated pollen was fixed in 5% glutaraldehyde in 66 mM PIPES buffer (pH 7.0) for 1 h directly on germination slides, and then transferred to microfuge tubes. Pollen grains were subsequently rinsed in PIPES buffer, post-fixed in 1% OsO_4 or 1.5 h, rinsed again in PIPES buffer, dehydrated through an acetone series, and embedded in Spurr's resin. Thin sections (90 nm) were post-stained with uranyl acetate and lead citrate and examined on a Zeiss EM 906. To measure the percentage of pollen grains containing body/stalk cell complexes, 1 μm serial sections were collected, stained with toluidine blue, and examined and photographed through a light microscope.

Results

When pollen is shed in the spring, the generative cell in *Picea abies* has already divided to produce the spermatogenous body cell and the sterile stalk cell (Fig. 1). Both the body and stalk cells contain numerous amyloplasts, which are much smaller than the amyloplasts in the surrounding vegetative cell. The completion of generative cell division into the body and stalk cells is very consistent. In 1 μm thick serial sections of 61 germinated pollen grains, every grain (100%) contained a body cell–stalk cell complex, and none contained only a generative cell. Since the stalk cell also consistently remains attached to the intine with the body cell oriented more towards the center of the grain (Singh 1978), one can easily distinguish the body cell and stalk cell in confocal sections by step focusing through the entire pollen grain. Step focusing was always carried out to ensure that images of the body cell were correctly identified and not confused with the stalk cell. In addition, step focusing ensured that microfilament bundles identified within the body cell were not confused with microfilament bundles in the surrounding vegetative cell.

The vegetative cell surrounding the body cell contains a complex array of rhodamine-phalloidin-labeled actin microfilaments throughout the cytoplasm, and the microfilament bundles are concentrated in a mesh which surrounds the body cell (Fig. 2). The body cell also contains a complex network of actin microfilament bundles which traverse the entire cytoplasm around the nucleus (Fig. 3). Microfilament bundles are clearly seen within the body cell in single, 0.5 μm thick optical sections (Fig. 4). Microfilaments within the body cell form a network just beneath the plasma membrane, which is parallel to the encapsulating network in the surrounding vegetative cell (Fig. 5). The microfilament network within the body cell is extensive. Microfilament bundles branch and twist around the small amyloplasts and organelles within the body cell (Fig. 6) and are in direct contact with the body cell nuclear surface (Fig. 7). The fluorescent labeling observed in treated pollen grains is not present in controls, where only the autofluorescence of the pollen grain wall was detected (Fig. 8).

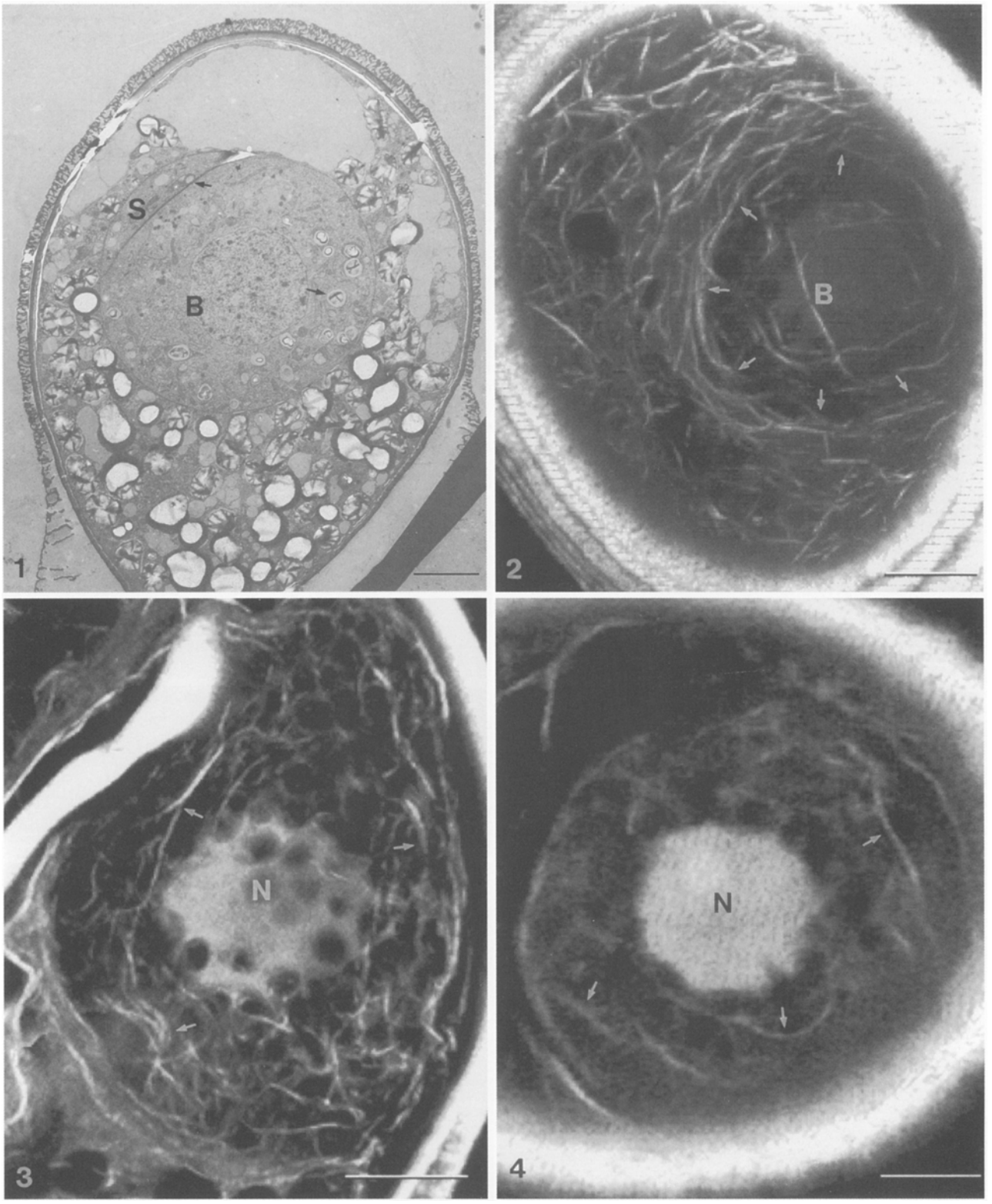
To confirm the presence of microfilaments in the spermatogenous body cell, actin was immunolabeled with a mouse monoclonal antibody to actin and a Cy3-conjugated secondary antibody. Microfilaments were detected in the body cell by this method. Discrete microfilament bundles are seen in the body cell cytoplasm surrounding the nucleus (Fig. 9). The stalk cell, the sterile mitotic sister to the spermatogenous body cell, also contains actin microfilaments which enmesh organelles and the nucleus (Fig. 10). When the primary antibody was omitted, only a faint fluorescence of the body cell nucleus and cytoplasm was detected (Fig. 11). Figures 9–11 were captured and processed with identical confocal settings.

Fig. 1. The generative cell has already divided into the body cell (*B*) and stalk cell (*S*) when pollen is released in *Picea abies*. The stalk cell is always closest to the pollen grain wall while the body cell is oriented more towards the center. Both the body cell and stalk cell contain numerous amyloplasts (arrows), which are much smaller than the amyloplasts in the surrounding vegetative cell

Fig 2. An extensive network of microfilament bundles is found throughout the vegetative cell of the pollen grain. In this projection of seven optical serial sections, the dense network of actin bundles (arrows) forms a spherical basket which enmeshes the body cell. Microfilament bundles are also present within the body cell (*B*)

Fig. 3. The spermatogenous body cell contains a dense array of microfilament bundles. In this projection of four 0.5 μm thick optical serial sections, microfilament bundles (arrows) traverse the body cell cytoplasm beneath the plasma membrane, throughout the cytoplasm, and around the nucleus (*N*). Nucleus labeled with propidium iodide

Fig. 4. A single optical section (0.5 μm thick) making a median slice through the body cell shows that the microfilament bundles (arrows) are within the body cell cytoplasm around the nucleus (*N*). Nucleus labeled with propidium iodide. All bars: 10 μm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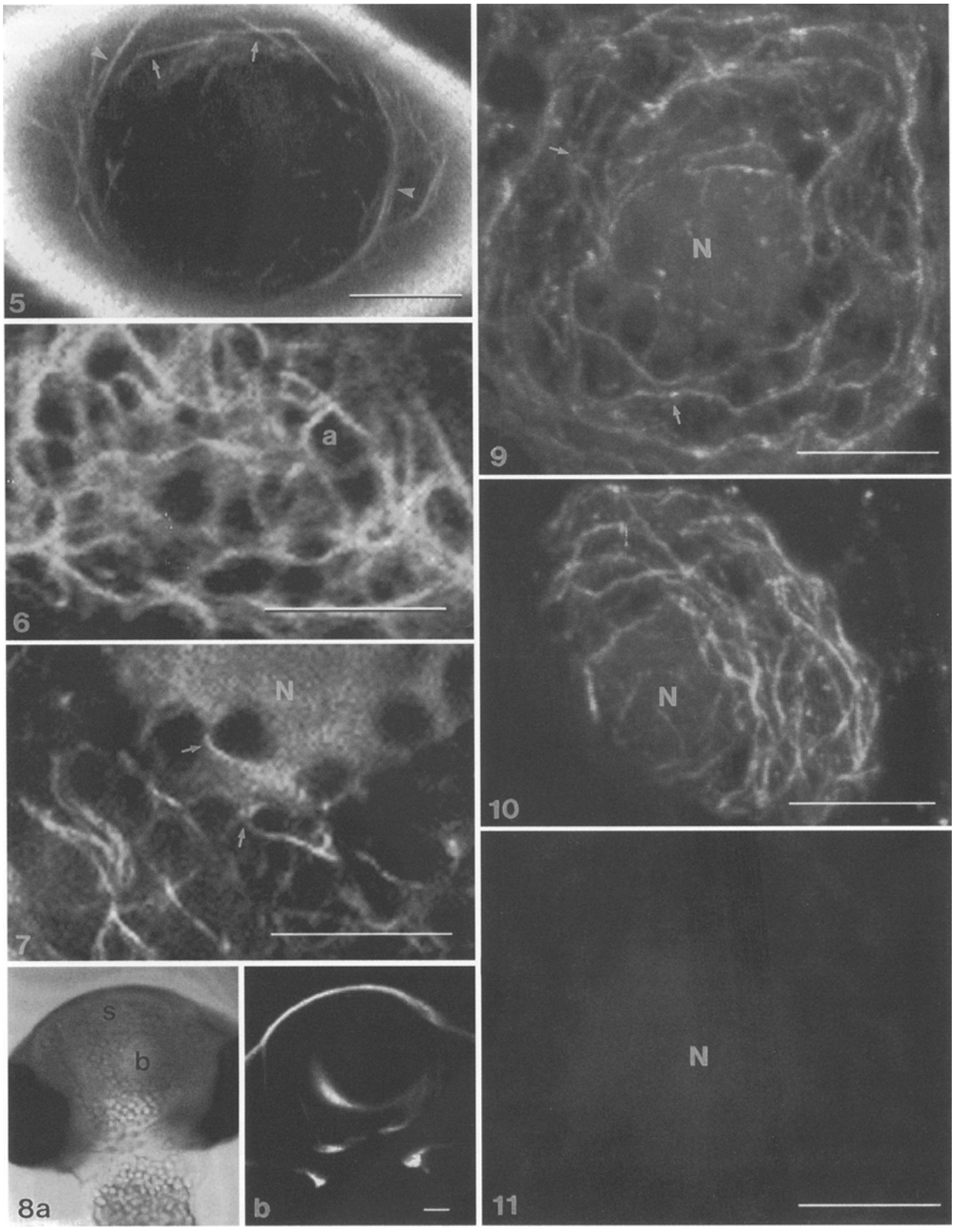
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Discussion

A complex network of actin microfilament bundles was identified within the vegetative cell, body cell, and stalk cell in *Picea abies* pollen grains. Within the vegetative cell, microfilament bundles traverse the entire cytoplasm but are enriched in a spherical network which surrounds the body cell, and the bundles in this network are parallel with microfilaments in the body cell. Although microfilaments form a dense network in the vegetative cell of many angiosperms (Heslop-Harrison et al. 1986; Pierson 1988; Tiwari and Polito 1988, 1990; Heslop-Harrison and Heslop-Harrison 1992a, b), a concentrated organization of microfilaments around the generative cell has only been reported in *Brassica napus* (Hause et al. 1992) and *Hyacinthus orientalis* (Heslop-Harrison and Heslop-Harrison 1992a). The current angiosperm model suggests that the generative cell moves into the pollen tube using primarily an actin-myosin system (Pierson and Cresti 1992, Russell 1996). Myosin is present on the generative-cell surface (Heslop-Harrison and Heslop-Harrison 1989, Tang et al. 1989a, Miller et al. 1995, Tirlapur et al. 1995, Bohdanowicz et al. 1995) and translocates pollen tube organelles in vitro (Kohno et al. 1990). The dense concentration of microfilaments around the body cell of *P. abies* seen in the present study, as well as the structural arrangement of microfilament bundles in long arrays emerging from the pollen grain aperture and extending down the tube in *P. abies* (Lazzaro 1996), *Pinus sylvestrus* (de Win et al. 1996), and *Pinus densiflora* (Terasaka and Niitsu 1994) suggest that the migration of the body cell in vivo may be directed along microfilaments. In *P. abies*, pollen grains land in the pollination droplets on female cones in the spring and germinate within a cleft in the micropyle. During the next 1–2 weeks, pollen tubes grow 100–200 μm through the integument and neck cells to reach the archegonium. After the pollen tube tip has reached the archegonium, there is a second period of 1–2 weeks when the body cell and stalk cell separate from the intine within the grain, migrate into the tube, and the body cell divides to produce two sperm nuclei (Christiansen 1972). This cell division is incomplete in *Picea* spp., since only a partial cell plate forms between the two sperm nuclei in *Picea glauca* (Dawkins and Owens 1993). When *Picea* pollen is germinated in vitro, the body cell and stalk cell remain within the grain (Dawkins and Owens 1993, Lazzaro 1996) so at present we are not able to directly observe the migration of the body cell into the tube nor the formation of sperm nuclei in vitro.

This study indicates that actin microfilaments are present within the body cell of conifers. These microfilament bundles form a complex network in the body cell which enmeshes the small amyloplasts, other organelles, and the body cell nucleus. Microfilament bundles branch along the body cell nuclear surface. Microfilament bundles also form a spherical array in the body cell cortex, beneath the plasma membrane, and this array is parallel to microfilaments in the surrounding vegetative cell. The presence of microfilaments in this spermatogenous body cell is in contrast

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Fig. 5. In this single, 0.5 μm thick optical section, microfilament bundles along the periphery of the body cell (arrows) form a network beneath the plasma membrane. This network is parallel to the microfilament bundles in the surrounding vegetative cell (arrowheads) which enmesh the body cell

Fig. 6. Microfilament bundles within the body cell cytoplasm branch and twist around small amyloplasts (*a*) which appear as dark ellipsoids in this single, 0.5 μm thick optical section

Fig. 7. Microfilament bundles (arrows) are coincident with the nuclear surface (*N*) in this single, 0.5 μm thick optical section through the body cell. Nucleus labeled with propidium iodide

Fig. 8. b In controls which were fixed and permeabilized in buffer lacking rhodamine-phalloidin and propidium iodide, only the autofluorescent pollen grain wall is detected. The curved line across the vegetative cell is actually a cleft in the pollen grain wall. **a** DIC image; the body (*b*) and stalk (*s*) cells are visible within the pollen grain

Fig. 9. Microfilament bundles were detected within the spermatogenous body cell with monoclonal antibodies to actin coupled to Cy3-labeled secondary antibodies. Distinct microfilament bundles traverse the body cell cytoplasm (arrows) and form a network around the nucleus (*N*) in this projection of 7 optical serial sections

Fig. 10. Microfilament bundles are also present in the sterile stalk cell, the mitotic sister of the spermatogenous body cell. The extensive network throughout the cytoplasm surrounds the nucleus (*N*) in this projection of 8 optical serial sections

Fig. 11. In controls where the primary antibody was omitted, faint fluorescence is detected in the body cell nucleus (*N*) and cytoplasm. Figures 9–11 were collected and processed with identical confocal settings. All bars: 10 μm

to the prevailing hypothesis in literature that microfilaments are absent in the spermatogenous generative cells of angiosperms (reviewed in Palevitz and Tiezzi 1992). However, there is evidence which conflicts with this hypothesis. Microfilaments were reported in the generative cell of *Rhododendron laetum* (Taylor et al. 1989), but this work could not be replicated by others (Palevitz and Liu 1992). In a more recent review, microfilaments were reported in the generative cell of *Lilium longiflorum* (Knox et al. 1993). There is also recent evidence that the microfilament motor protein myosin is present within the generative cell of *Nicotiana tabacum* (Tirlapur et al. 1995, 1996) and that the generative cell of *Ledebouria socialis* may contain g-actin together with profilin (Hess et al. 1995). In conifers, microfilaments and myosin were both reported in the generative cell of *Pinus densiflora* pollen (Terasaka and Niitsu 1994), although it is not clear from the images presented whether the microfilaments are inside the generative cell or surrounding it in a network within the vegetative cell.

Microfilament bundles were also identified in the stalk cell of *P. abies*, forming a network around the organelles and nucleus similar to that seen in the body cell. The sterile stalk cell and fertile body cell are the result of an equal mitosis of the generative cell in conifers (Singh 1978). There is no apparent exclusion of microfilaments following the mitotic division which produces the spermatogenous cell. This is in contrast to angiosperms, where microfilaments are present through unequal mitosis of the microspore (Brown and Lemmon 1991a, 1992; Dinis and Mesquita 1993) but are excluded from the resulting spermatogenous generative cell and only found in the resulting vegetative cell (Brown and Lemmon 1991b).

If conifers differ from angiosperms in the presence of microfilaments in the spermatogenous cell, then there should be a functional reason for this distinction. Plastid DNA is paternally inherited in most conifers (Owens and Morris 1990), so microfilaments in the body cell may organize the plastids so they are correctly sorted following the formation of two sperm nuclei. In *P. glauca*, the body cell nucleus divides, but an incomplete cell plate forms, producing a cell with two sperm nuclei (Dawkins and Owens 1993). When the pollen tube penetrates into the egg cell in *Picea* spp., one sperm nucleus migrates 500–1000 μm to the center of the massive cell together with a surrounding layer of sperm plastids, while the other sperm nucleus remains at the top of the egg cell or associates with the ventral canal cell (Singh 1978). Microfilaments may be necessary in the body cell so that following mitosis, the functional sperm nuclei will move through the egg cell with its complement of plastids intact and held in place by the actin cytoskeleton. Even though the majority of evidence in literature indicates that angiosperm generative cells and sperm lack microfilaments (reviewed in Palevitz and Tiezzi 1992), it might be worthwhile to continue searching for microfilaments in the generative cells and sperm of angiosperms which exhibit paternal plastid inheritance (see Corriveau and Coleman 1988).

There must be a functional reason for actin microfilaments in the spermatogenous body cell. However, the role of microtubules cannot be ignored. In the angiosperms, microtubules are clearly involved in the organization and function of generative cells and sperm, and may form the only cytoskeletal network in these cells (Palevitz and Tiezzi 1992). In Pinaceae, microtubules have been identified in the generative cell of *Pinus densiflora* (Terasaka and Niitsu 1994) and in the body cell of *P. glauca* (Dawkins and Owens 1993). Microtubules form a complex network in the vegetative, body, and stalk cells of *P. abies* (Lazzaro unpubl. obs.), so some of the functions postulated for microfilaments in the present study may be shared by microtubules.

Microfilaments were identified within the body cell by two methods. Rhodamine-phalloidin is a common marker for filamentous actin, and the specific protocol employed here preserves the detailed organization of microfilament networks (Lazzaro 1996). The actin antibody used to establish a second line of evidence for microfilaments in the body cell is known to react with plant actin in angiosperm pollen tubes (Tang et al. 1989b, Åström et al. 1991, Sorri et al. 1996). The lack of fluorescent signal in controls from both methods indicates that the filamentous structures observed with rhodamine-phalloidin and Cy3-conjugated actin antibodies are microfilament bundles. The presence of microfilaments within the spermatogenous body cell is a fundamental difference between pollen development in conifers and angiosperms.

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