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Pollen Tube Cytoskeleton: Structure and Function

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Abstract

This review deals with the structure and function of the pollen tube cytoskeleton from pollen germination to the formation of two sperm cells by division of the generative cell in the tube. The structure and function of microfilaments (MFs) and microtubules (MTs) are described in the binucleate/bicellular pollen tubes of *Nicotiana tabacum.* In this species the pollen grain and tube are formed of two cells; the vegetative cell (VC) enclosing the generative cell (GC). After pollen germination the VC forms the pollen tube, and the vegetative nucleus (VN) and GC move in the early phase of tube elongation to the tube tip, where they stay in tight association until division of the GC takes place. In this review we discuss the function of MFs, actin binding proteins (ABPs), MTs, and microtubule-associated proteins (MAPs and motor molecules) in growth and in the movement of cell organelles in the tube. Special attention is paid to the description of GC division and to consideration of factors that could cause GC cell cycle arrest during the early phase and induce GC division in the late phase of tube elongation. The movement of the VN and GC close to the tube tip can be thought to divide the function of the tube roughly into two parts: a tip part that contains the secretion machinery for primary wall formation and growth, with highly efficient exo- and endocytotic pathways, and a basal part behind the VN and GC, where secretion is more directed to secondary wall thickening, callose plug formation, and vacuolar biogenesis. The local calcium concentration seems to be an important factor determining the structure and function of the cytoskeletal elements in the tip region and in the basal part of the tube. In the future, *in vivo* or semi- *in vivo* cultured pollen tubes need to be used to test whether cytoskeletal functions deduced from the *in vitro* experiments occur also during *in vivo* tube growth.

Key words: Endocytosis; Exocytosis; Generative cell division; Microfilaments; Microtubules; Pollen tube growth; Vacuolar biogenesis

Binucleate and Trinucleate Pollen Grains

The majority of flowering plants, including *Nicotiana tabacum,* investigated by the authors, release binucleate/bicellular pollen; the pollen contains a large vegetative cell (VC) enclosing a generative cell (GC). The GC divides during pollen tube growth forming two sperm cells (Figure 1a). In trinucleate/tricellular grains, GC division takes place already in the pollen grain, resulting in the release of pollen with two sperm cells in the VC (Figure 1b). Out of the almost 2000 flowering plant species studied, 70% have binucleate and 30% have trinucleate pollen grains. Binucleate pollen grains have better storage longevity, viability, and germination capacity

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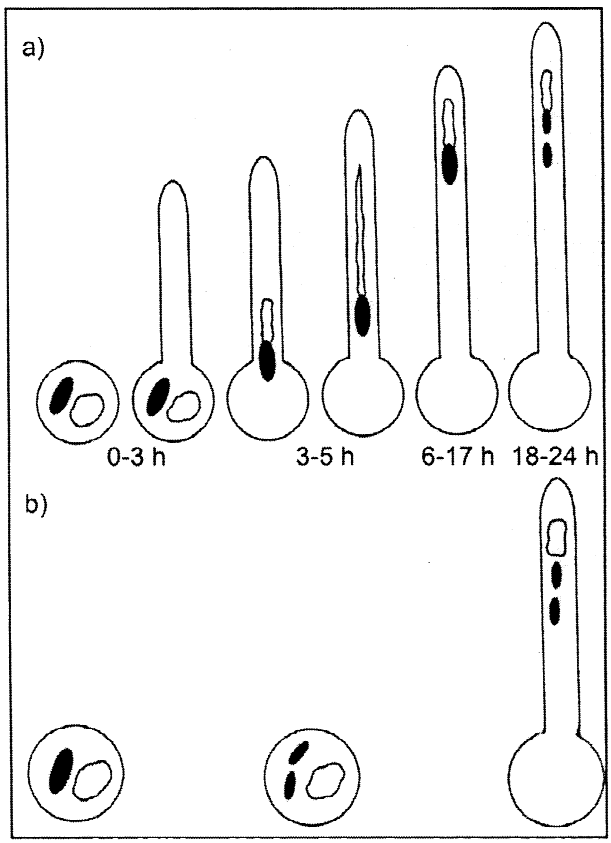

Figure 1. A schematic drawing showing the differences between binucleate **(a)** and trinucleate **(b)** pollen grains and tube structure. The GC / sperm cells are in *black* and the VN is in *white* in the drawing. In binucleate pollen **(a)** the GC moves into the basal regions of the tube in close proximity to VN only after the tube has reached a certain length. During the movement towards the tip region of the tube the VN is highly extended. At the tube tip the VN and GC remain tightly associated. After several hours of tube elongation the GC divides forming two sperm cells. The time sequences for the different phases of VN-GC movement is indicated in **(a)** for *Nicotiana tabacum* pollen tubes grown under *in vitro* conditions. In trinucleate pollen **(b)** the GC divides in the grain to form two sperm cells, which are transported from the grain into the growing tube.

than trinucleate grains (Brewbaker 1967). The delay in germination before growth of the pollen tube starts *in vitro* is generally considerably longer for binucleate than for trinucleate pollen. In *Nicotiana alata* germination occurs slowly, and during pollination it takes at least 48 h for the pollen tube to reach the ovule (Hoekstra and Bruinsma 1978). In trinucleate species pollen germination occurs rapidly, the rate of tube growth is extremely high, and fertilization can be accomplished within 30 min (Hoekstra and Bruinsma 1978; Hoekstra and Bruinsma 1979; Mascarenhas 1993). It has been suggested that pollen tubes possess an early autotrophic growth period, utilizing internal pollen resources, and a heterotrophic, rapid growth period, using style exudates (Rosen 1971), or nutrients from the growth media under *in vitro* conditions. The initial growth of binucleate pollen tubes would represent the autotrophic growth period, and the heterotrophic growth period would be characterized by an increased growth rate, callose plug formation, and GC mitosis (Derksen and others 1995b).

During *in vitro* culturing of *N. tabacum* pollen tubes three important phases can be distinguished (Åström 1997): (1) germination of the pollen grains and movement of the GC and vegetative nucleus (VN) into the short tube, (2) extensive elongation of the tube including vacuolization and callose plug formation in the basal part of the tube, and (3) division of the GC into two sperm cells. Cytoskeletal elements, particularly microtubules (MTs) and microfilaments (MFs), have been shown to be important for all these processes (Laitiainen 1998; Åström and others 1991; Åström and others 1995). The reorientation of the cytoskeletal structures during different phases of pollen tube growth suggests that MTs and MFs respond to intra- and extracellular signals.

Actin Cytoskeleton and Pollen Tube Growth

MFs and MTs have long been recognized as structural components of the pollen tube cytoplasm (Franke and others 1972), but only after immunocytochemical and related research methods were developed for plant cells (Wick and others 1981) it became possible to observe the distribution patterns of MFs and MTs in pollen tubes grown *in vitro* and *in vivo* (Derksen and others 1985; Pierson and Cresti 1992; Pierson and others 1986; Raudaskoski and others 1987). Pollen tube MFs visualized with fluorescent phalloidin staining or with indirect immunofluorescence (IIF) microscopy are mainly longitudinally orientated and occur both in the cortical and central cytoplasm of the pollen tubes. Similar MF distribution has recently been confirmed from living pollen tubes in which MFs were visualized by the transient expression of the MF-binding small protein, talin, labelled with green fluorescent protein (GFP) (Kost and others 1998).

In the tip region of *Nicotiana* pollen tubes, the MFs appear as delicate structures whereas thick MF bundles are found in the basal part of the tube (Åström and others 1991; Åström and others 1995).

The occurrence of MFs in the apical tip region was recently questioned after microinjection of small amounts of fluorescent phalloidin into living pollen tubes of *Lilium longiflorum* revealed several longitudinally orientated MF bundles in the basal part of the tube, but no MFs in the outermost tip region. Ultrastructural studies of freeze-substituted pollen tubes confirmed these observations (Miller and others 1996). Single MFs occur next to the plasma membrane within 5 μm from the tip, whereas MF bundles first appear 10–20 μm from the tip. The absence of MFs at the tip of the growing tube is believed to be due to the tip-focused Ca^{2+} gradient (Miller and others 1996), indispensable for the growth of the tube (Malhó and others 1994; Malhó and others 1995; Malhó and Trewavas 1996; Messerli and others 1999; Pierson and others 1994; Pierson and others 1996). The MF-free region in the outermost part of the pollen tube tip is difficult to visualize by regular methods such as fluorochrome-labelled phalloidin staining of MFs in living or rapidly fixed cells or by IIF microscopy with actin antibodies (Åström and others 1991; Åström and others 1995) even when the pollen tubes are quick-frozen and freeze substituted.

In *N. tabacum* (Derksen and others 1995a) and *L. longiflorum* pollen tubes (Lancelle and others 1997) the cell wall of the extreme tip appears to be slightly thicker than the cell wall below the tip region. The former region could correspond to the region with slow expansion and low curvature, recently shown to occur in the tip of root hairs of *Medicago truncatula* (Shaw and others 2000). The maximum growth rate and curvature in the growing root hairs are achieved in the annular zone surrounding the slower growing apical dome (Shaw and others 2000), which agrees with the occurrence of the thin wall layer region below the extreme tip in *Nicotiana* pollen tubes.

Depolymerization of MFs with cytochalasins gradually stops *Nicotiana* pollen tube growth (Figure 2) indicating that MFs are indispensable for polarized growth of the pollen tube (Laitiainen 1998). Cytoplasmic streaming with a reverse-fountain pattern is a typical feature of young growing pollen tubes. In this pattern the cytoplasmic streaming is orientated towards the tip in the cortical region and toward the base in the subcortical part of the tube, and a change in the direction of streaming takes place below the tip region of the tube (Derksen and others 1995a; Derksen and others 1995b; Heslop-Harrison and Heslop-Harrison 1987). Treatment of pollen tubes with cytochalasins has shown that depolymerization of MFs stops the cytoplasmic streaming, which suggests that the MFs provide the tracks for the streaming (Franke and others 1972; Kohno and Shimmen 1988; Perdue and Parthasarathy 1985). Cytoplasmic streaming is thought to be involved in vesicle transport towards the tip but the function of the reverse streaming is still unclear.

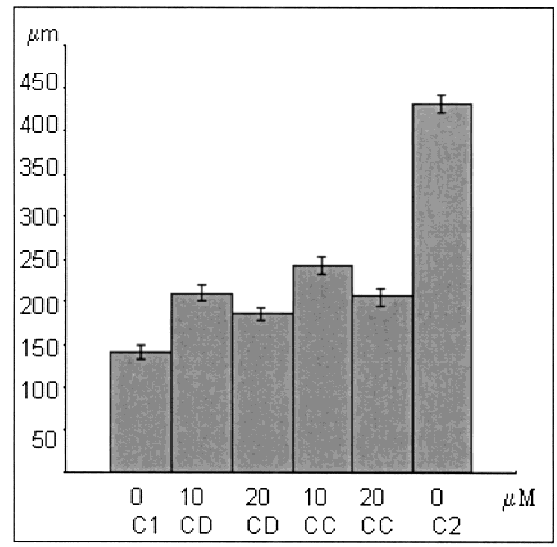

Figure 2. The effects of cytochalasin D and C on *Nicotiana tabacum* pollen tube elongation. Pollen tubes were cultured for 3 h on control medium (C1) and then transferred to cytochalasin (CD or CC) or new control media (C2) for another 3 h CC = cytochalasin C, CD = cytochalasin D. The tube lengths (μm) and cytochalasin concentrations (μM) are indicated in the figure. n = 50 for each column.

The terminal 10–15 μm region of the tube, devoid of MFs (Miller and others 1996), is mainly occupied by vesicles (Derksen and others 1995a; Lancelle and others 1997) which are assumed to contain precursors for cell wall synthesis during the polarized growth of the pollen tube. Ultrastructural studies have shown that the appearance of vesicles next to Golgi complexes and in the tip region in the subapical parts of the tube is often very similar (Figure 3a, b), and rough endoplasmic reticulum (ER) is observed in close proximity to the Golgi complexes (Figure 3c). This suggests that pollen tubes contain a secretory pathway similar to that described in yeast and animal cells, in which extracellular proteins are synthesized on rough ER, modified posttranslationally in the lumen of ER and carried to *cis* Golgi for further modifications, and finally delivered as secretory vesicles from the *trans* Golgi network to the plasma membrane. In plants, immunolocalization with antibodies against different carbohydrate groups has revealed that the synthesis and assembly of cell wall matrix polysaccharides also takes place in the Golgi complex. The backbone of pectin is assembled in *cis* and *medial* Golgi and the side chains added in the *trans* Golgi network, and the synthesis

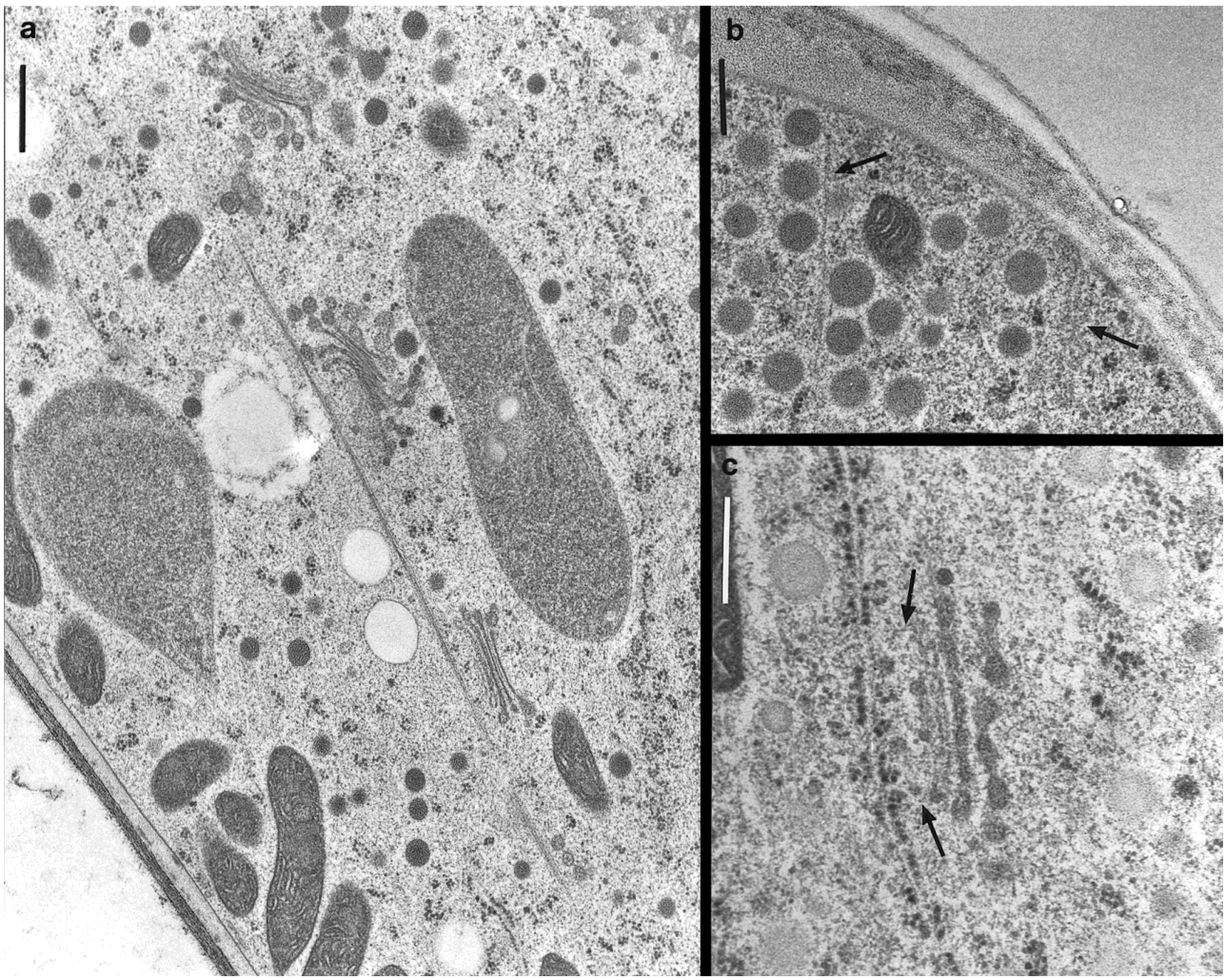

Figure 3. Ultrastructure of quick-frozen and freeze substituted pollen tubes of *Nicotiana tabacum.***(a)** Golgi complexes in close association to MFs in the subapical part of the tube. **(b)** Vesicles seen along MFs in the tip region of the tube **(c)** A Golgi complex next to the endoplasmic reticulum. Arrows point to the region with small vesicles between ER and *cis* Golgi. Bar in **(a)** is 0.5 μm, in **(b)** and **(c)** 0.3 μm.

of hemicelluloses occurs in *trans* Golgi and the *trans* Golgi network. The assembled polysaccharides are then transported in secretory vesicles to the plasma membrane (Driouich and others 1993).

Exo- and Endocytosis and Actin Cytoskeleton

The pollen tube wall in *N. tabacum* and *N. alata* consists of 86% callose, 5% cellulose, 4% arabinan, and 5% galacturonan (Li and others 1999). A callose synthase (CalS) enzyme, different from the wound- and Ca^{2+}-activated CalS from other plant tissues, has been characterized from *Nicotiana* pollen tubes (Li and others 1999; Schlüpmann and others 1993). The synthesis of CalS enzyme starts at pollen germination, and the enzyme is transported as a zymogen from the site of synthesis to the plasma membrane where it is activated by proteolytic cleavage (Li and others 1999). Exocytosis of vesicles containing CalS zymogen is presumed to take place either at the tube tip or in the subapical part at the site of callose accumulation (Li and others 1999), corresponding to approximately 30 μm behind the tip region (Ferguson and others 1998). If exocytosis of CalS were to take place at the very tip, it would need to remain inactive until the vesicle fusion site had moved a distance away from the extreme tip as a consequence of tip growth (Li and others 1999). In pollen tubes, cellulose is detected about 5–15 μm behind the growing tube tip, and it colocalizes with callose in the inner, electron-lucent layer of the pollen tube wall (Ferguson and others 1998). As in other plant cells, cellulose is probably synthesized at the cell surface (Delmer 1999), also requiring the

transport of cellulose synthase units to the plasma membrane.

Immunolabeling with antibodies specific for methyl-esterified pectin and acidic pectin has confirmed that the cell wall in the tip region consists of the highly plastic methyl-esterified pectin (Li and others 1994). Below the tube tip the methyl-esterified pectin is de-esterified to acidic pectin, which readily interacts with Ca^{2+} ions, increasing the rigidity of the pectin network and pollen tube wall behind the apex (Derksen 1996). The secretory vesicles closest to the tube tip must thus contain a load of pectin and enzymes for pectin modifications. The wide variety of products in the secretory vesicles needed for pollen tube wall formation raises the question of whether the Golgi complexes in the pollen tube all have the same function, or if some of them could be specialized in pectin production and others in glycosylation of secreted proteins such as callose and cellulose synthases. A single type of secretory vesicle containing all the different wall components, enzymes, and secretory proteins has been described in tobacco (Derksen 1996; Derksen and others 1995a), whereas in pollen tubes from *Brugmansia suaveolens,* different types of Golgi-derived vesicles are observed (Geitmann and others 1995), leaving the question open.

For a long time it has been evident that endocytosis also must play a role in pollen tube growth. It has been calculated that an excess of membrane material is transported to the tip of the pollen tubes during exocytosis, which requires endocytotic plasma membrane recycling to maintain the tube tip structure (Derksen and others 1995a; Picton and Steer 1983; Steer 1988). The functioning of the endocytotic pathway was supported by the presence of a high number of coated pits and vesicles carrying a clathrin-like coat in the tip area of tobacco pollen tubes (Derksen and others 1995a). Isolation of a soybean cDNA highly homologous to animal clathrin heavy chain proved that clathrin is expressed in plant cells (Blackbourn and Jackson 1996). Polyclonal antibodies against purified soybean clathrin vesicles and used in isolation of the clathrin heavy chain cDNA strongly labelled the first 10 µm of the tip region of *L. longiflorum* pollen tubes, which was in agreement with the observation of a high number of coated pits and vesicles in the same area in ultrastructural studies (Derksen and others 1995a).

The relationships between exocytosis, endocytosis, and the actin cytoskeleton in the growing pollen tube tip make an interesting question to which no clear answer can yet be provided. The absence of MFs or their extremely low number (Miller and others 1996) in the region with high accumulation of secretory vesicles could be related to the need to remove the rigid cortical actin cytoskeleton from the tube tip. In animal cells, cortical actin is thought to be inhibitory to exocytosis (Valentijn and others 1999). If the same is true in pollen tubes, visualization of a MF network at the tube tip with conventional immunocytochemical and related methods (Åström and others 1991; Åström and others 1995) would be a sign of unsuccessful staining, resulting in rearranged actin structure at the tip. It could also be that the pollen tubes with strong actin staining at the tip represent those with less effective growth rates. In higher filamentous fungi the visualization of MFs with rhodamine phallacidin in the hyphal tips of fast-growing saprophytic fungi is always poor, whereas in the hyphal tips of the slow-growing ectomycorrhizal fungi, an intense staining of MFs is obtained (Gorfer and others 2001; Salo and others 1989). In the tip region of slow-growing hyphae, the prominent actin cytoskeleton might be involved in keeping the shape of the tip in the absence of fast cell wall formation. The occurrence of MFs in close proximity to coated pits and coated vesicles in the tip region containing numerous Golgi complexes (Derksen and others 1995a) suggests that in pollen tubes there could be a connection between endocytosis and the actin cytoskeleton, as perhaps is the case in animal cells (Qualmann and others 2000). As part of the control of cytoplasmic streaming, MFs below the tip may force the secretory vesicles into the V-shaped accumulation at the tube tip. This could take place by focusing the vesicle traffic from the numerous surrounding Golgi complexes towards the tip and by regulating the positioning of the Golgi complexes in respect to the secretory vesicle accumulation.

Actin Binding Proteins

Immunological, cell, and molecular biological and physiological research methods have identified in pollen tubes a few proteins homologous to those that regulate the structure and function of the actin cytoskeleton in animal and yeast cells. Monoclonal and polyclonal antibodies (Miller and others 1995; Tang and others 1989; Tirlapur and others 1995) raised against myosins I, II, and V from animal cells, visualized myosin in the pollen tubes of *L. longiflorum* and *N. alata.* In *L. longiflorum,* myosin I was localized to the plasma membrane, to the surface of the VN and GC, and was seen as spots in the pollen tube cytoplasm. The visualization of myosin on the VN and GC surface suggests that myosin I could be involved in the unidirectional slow movement of the VN and GC towards the tube tip (Miller and others 1995). Antibodies against myosin II subfrag-

ment (S1) and light meromyosin (LMM) visualized myosin II in a pattern that seemed to follow the MF tracks of the pollen tube. Similarly the spots visualized with the antibody against myosin V could be thought to follow the MF tracks (Miller and others 1995). The function of different myosins in intracellular pollen tube traffic awaits molecular biological data; to date no genes encoding myosins appear to be cloned from *L. longiflorum* or *Nicotiana* species.

Several genes encoding the small actin binding proteins (ABPs) cofilin and profilin have been characterized from plants, including maize genes for pollen specific cofilin (Lopez and others 1996) and profilin (Gibbon and others 1998; Staiger and others 1993) and a profilin gene from tobacco (Mittermann and others 1995). Cofilin and profilin are both small polypeptides, which in animal cells are suggested to have similar and opposite effects on MF dynamics (Bamburg 1999). Cofilin belongs to the ADF (actin depolymerizing factor) protein family, the members of which interact with actin monomers and filaments in a pH-sensitive manner. When ADF/cofilin binds to filamentous (F) actin it induces a change in the helical twist and fragmentation, and accelerates the dissociation of subunits from the pointed ends of actin filaments (Bamburg 1999; Cooper and Schafer 2000). The properties of plant cofilin have been analyzed by using recombinant proteins from the maize pollen specific cofilin gene *ZmADF1* (Hussey and others 1998) and from *ZmADF3,* which is suppressed in pollen but expressed in all other maize tissues. ZmADF3 has the ability to bind monomeric (G) actin and F-actin and to decrease the viscosity of polymerized actin solutions indicating an ability to depolymerize actin filaments (Lopez and others 1996). ZmADF3 is phosphorylated on Ser6 by a calcium-stimulated protein kinase in plant extracts (Smertenko and others 1998). This suggests that phosphorylation and dephosphorylation could regulate actin binding ability of cofilin and thus affect actin cytoskeleton stability, as is the case in animal cells (Daniels and Bokoch 1999). Dual visualization of actin and ZmADF3 with polyclonal antibodies raised against recombinant ZmADF3 revealed a change in orientation of MFs towards the emerging root hair at the same time as ZmADF3 became concentrated at the tip, implying a role for ZmADF3 in actin remodelling (Jiang and others 1997). Microinjection of recombinant ZmADF1 protein in stamen hair cells of *Tradescantia blossfeldiana* led first to cessation and then to recovery of cytoplasmic streaming although the streaming changed from its original longitudinal to transverse direction (Hussey and others 1998). Staining of the microinjected cells 45 min later with fluorescein-conjugated phalloidin showed that the longitudinal MF cables typical of control cells had been replaced by transverse MF cables. These experiments suggest that the cofilins encoded by pollen tube-specific genes are probably also involved in regulation of MF dynamics, although this has not yet been confirmed by cofilin localization experiments in pollen tubes.

Profilin is a G-actin binding protein that is known to interact in animal and yeast cells with proline-rich motifs of other proteins and with polyphosphoinositides. The interaction of profilin with G-actin provides a mechanism to sequester actin monomers and promote actin depolymerization. It appears that profilin may also be involved in promoting actin polymerization. This might take place by binding to proline-rich motifs in proteins that convey intra- or extracellular signals to reorganization of actin cytoskeleton (Mullins 2000). The presence of several pollen-specific profilin isoforms in maize has raised the question of whether they are functionally unique or redundant (Gibbon and others 1997; Gibbon and others 1998). Microinjection of birch pollen-specific profilin into the stamen hair cells of *T. blossfeldiana* caused a rapid irreversible change in the cellular organization and cytoplasmic streaming due to depolymerization of MFs (Staiger and others 1994), and these results have been confirmed by the use of bacterially expressed maize pollen profilin isoforms in similar experiments (Gibbon and others 1997; Gibbon and others 1998). These results support the model that pollen profilins act as actin-sequestering proteins that promote actin depolymerization (Staiger and others 1994). Immunocytochemical experiments suggest that profilin is uniformly distributed all over the pollen tube (Vidali and Hepler 1997), which makes it difficult to assign a specific function for profilin in general or to the role of profilin isoforms in pollen tube growth and development. Recently it has been shown that one of the profilins expressed in pollen tubes shows higher proline-rich motif binding capacity than others (Gibbon and others 1998), and that the actin-sequestering activity of profilins is dependent on Ca^{2+}concentration (Staiger 2000). These are interesting observations that may help us to understand the function of pollen tube profilins.

Plants also have several genes with high homology to animal villin (Klahre and others 2000). The first plant villin was isolated from *L. longiflorum* pollen tubes as a 135 kD actin-bundling protein (Yokota and others 1998; Yokota and Shimmen 1999). Its identity as a villin-gelsolin family member (Vidali and others 1999) was confirmed by partial amino acid sequencing and by isolating the corresponding cDNA from a pollen grain cDNA expres-

sion library. Immunodetection of villin revealed its co-localization with MF bundles in pollen tubes. Due to the gelsolin-like headpiece, villin may also act as an actin-severing protein, although this activity has not yet been demonstrated for plant villins. Calcium and calmodulin together inhibit the actin-bundling activity of pollen tube villin (Yokota and others 2000), adding villin to the group of Ca^{2+}-regulated actin-binding proteins.

Microtubule Cytoskeleton

In *in vitro* culture of *N. tabacum* pollen tubes the movement of the VN and GC into the pollen tube takes place between 3 and 6 h after spreading of the pollen grains on growth medium; at this time the pollen tubes have reached lengths of 200–300 µm (Åström and others 1995). The structure and orientation of the MT cytoskeleton differs before and after the movement of the VN and GC into the tube. In germinating pollen tubes the MT tracks are delicate but become thicker after the VN and GC have reached their position in the apical part of the tube. Helical orientation and bundling of MTs increases behind the VN and GC complex close to the plasma membrane, whereas axial MTs occur in the central part of the tube and in the cytoplasm surrounding the VN and GC complex (Figure 4a–d). A common feature to all pollen tubes is the absence of MTs from the actual tip region and the short and randomly oriented MTs in the subapical region (Figure 4a).

In *N. tabacum* and other binucleate pollen tubes the GC is tightly associated with VN close to the tube apex. The GC is spindle shaped and long extensions of the GC are often seen to run along the surface and to protrude into the folds of the VN (Figures 4b, c), probably thus maintaining the association between GC and VN (Raudaskoski and others 1987). Ultrastructural analysis of serial sections of GC show five to six prominent longitudinally oriented MT bundles between the plasma membrane and the generative nucleus (GN), which close the GN in a cage-like structure (Raudaskoski and others 1987; Yu and Russell 1993). The MT bundles appear to be attached to the plasma membrane at each end of the GC (Figure 5a) and they can even extend into the apical extensions of the GC, where they could provide stiffness for the extensions necessary for extending along the surface and penetrating into the folds of the VN. MT bundles probably also control the spindle shape of the GN through attachments to the outer surface of the nuclear envelope (Figure 5a).

When the MTs in the GC are depolymerized either with cold treatment or the anti-MT drug oryzalin, the spindle shape of the GC and GN disappears and the GC disassociates from the VN (Åström and others 1991; Åström and others 1995). Isolation of GC from *N. tabacum* pollen grains (Theunis and others 1992) and other species (Tanaka and others 1989; Tanaka and Wakabayashi 1992) also leads to depolymerization of MTs and loss of the spindle shape of the GC. These observations confirm that the spindle shape of the GC and GN is dependent on the MT bundles in the GC. The depolymerization of the MT bundles in isolated GCs further suggests that the formation and maintenance of the MT bundles in the GC requires an interaction with the cytoplasm surrounding the GC in the pollen tube.

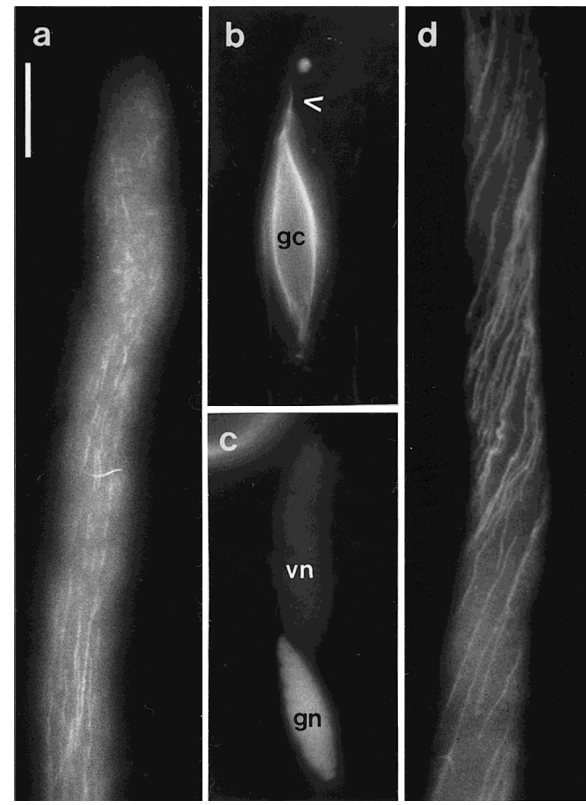

Figure 4. MT cytoskeleton of *Nicotiana tabacum* pollen tubes. **(a)** MTs are absent from the tube tip, short in the subapical region and uniform further down the tube. **(b–c)** MT bundles in GC (b). Note the MTs in the extension of GC (arrowhead) and the tubulin accumulation on the VN surface. DAPI staining of the VN (vn) and GN (gn) in the same tube in (c). **(d)** Helically arranged MT bundles behind the VN-GC complex. Bar 10 µm.

Microtubule-Associated Proteins

The MT bundles in the GC are the last ones to disassemble during cold or drug treatments, proving

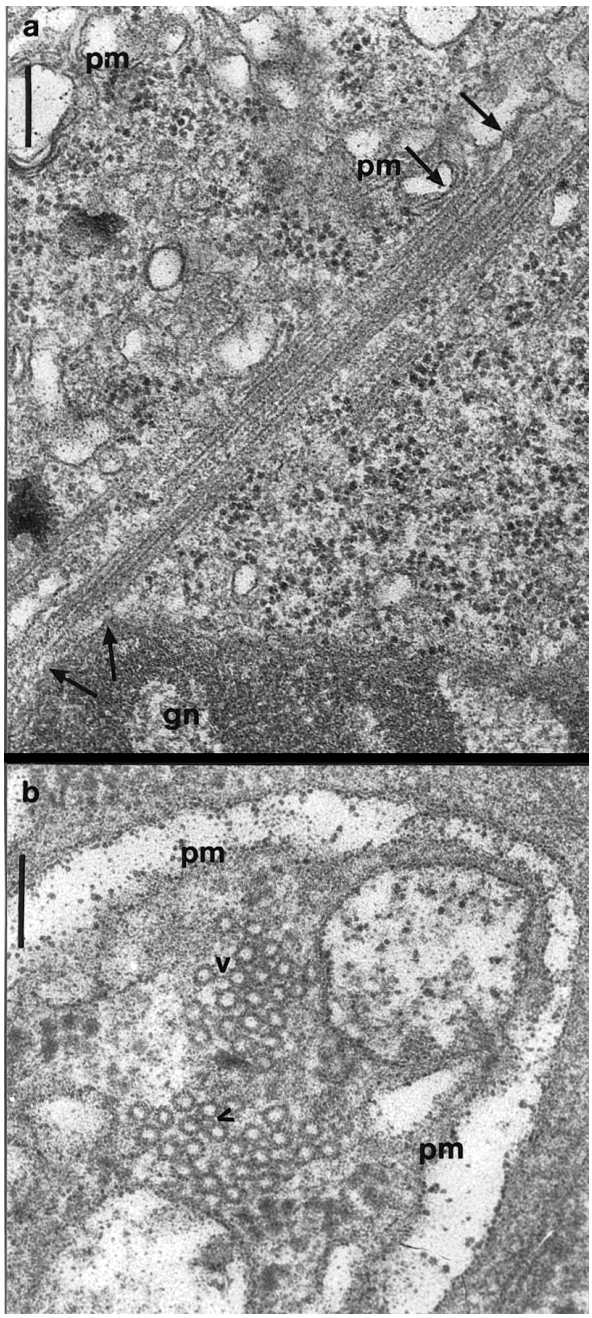

Figure 5. Ultrastructure of MT bundles in the GC cytoplasm of *Nicotiana tabacum* pollen tubes. **(a)** Note the close association of the MT bundle to the plasma membrane (pm) and to the nuclear (gn) envelope indicated by arrows. **(b)** Cross-section of a MT bundle in GC. Linkages between MTs are marked by arrowheads. The GC plasma membrane is indicated by pm. Bar in **(a)** is 0.2 μm and in **(b)** 0.5 μm.

that they are highly stable structures (Åström and others 1991; Åström and others 1995). The MT stability is probably due to cross-linking of the MTs by microtubule-associated proteins (MAPs) in the bundles (Figure 5b) and linking the bundles to the outer surface of the GN and to the GC plasma membrane (Figure 5a) (Palevitz and Tiezzi 1992; Raudaskoski and others 1987; Tiezzi 1991; Tiezzi and others 1988). Recently, transient expression of the MT binding domain of mammalian MAP4 labelled with GFP in leaf epidermal cells showed that this domain of MAP4 was able to bind to plant MTs (Marc and others 1998). It also showed that MAP4 especially induced bundling of longitudinally oriented MTs and that these bundles were relatively resistant to depolymerization by anti-MT herbicides. These observations agree well with the assumption that the stability of the MT bundles in the GC of pollen tubes is due to cross-linking of MTs with MAPs. In the experiment with the leaf epidermal cells the occurrence of highly bundled longitudinal MTs was interpreted, however, to represent an aberrant phenotype functionally impaired by the experimental procedure (Marc and others 1998).

Electron microscopical investigations of the GC (Palevitz and Tiezzi 1992; Raudaskoski and others 1987; Tiezzi 1991; Tiezzi and others 1988) reveal structures probably representing cross-bridges between individual MTs in the bundles and between MTs and the plasma membrane or the nuclear envelope (Figures 5a, b). Suspension cultured carrot cells (Chan and others 1996; Chan and others 1999; Cyr and Palevitz 1989), maize (Vantard and others 1991) and tobacco BY-2 cells (Jiang and Sonobe 1993) contain several polypeptides that induce bundling of MTs *in vitro.* One of the polypeptides is immunologically related to mammalian MAP tau (Vantard and others 1991). Elongation factor −1α (Ef-1α), necessary for protein translation, is also known to cause MT bundling (Durso and Cyr 1994). Immunocytochemical and related methods reveal MTs attached to the plasma membrane of protoplasts (Chan and others 1999). Treatment of plasma membrane ghosts deprived of MTs with cell extract from tobacco BY-2 cells showed that MTs polymerized from the plasma membrane of the ghost (Shibaoka 1994). These experiments suggests that polypeptides cross-linking MTs to the plasma membrane also exist in plant cells and probably will be found during future work on plant MAPs. No MAPs have yet been shown to occur in pollen tubes. The isolation of genes encoding putative plant MAPs (Smertenko and others 2000) will probably help to clarify the role of MAPs in organizing the MTs in special structures such as the prominent MT bundles in the GC of pollen tubes, and reveal the signals that regulate their organization.

MAPs also include motor molecules. In pollen tubes, four different MT-associated motor proteins

have been identified by biochemical and cell biological tools: two kinesins, a pollen tube kinesin homologue (PKH) (Cai and others 1996; Tiezzi and others 1992), and a 90 kD ATP-MAP (Cai and others 2000) and two dynein-related polypeptides (Moscatelli and others 1995; Moscatelli and others 1998). All polypeptides were isolated with taxol-stabilized bovine brain MTs. They have a molecular weight typical of kinesins and dyneins, show MT-stimulated ATPase activity, and the kinesins have been shown to induce MTs to glide in motility assays *in vitro.* These proteins are recognized both in immunoblotting and in IIF microscopy with antibodies prepared against conserved regions of animal kinesins and dyneins, and they appear to be located in the pollen tube cytoplasm but not in the GC. The PKH label occurred mainly in the tip region in *Nicotiana* pollen tubes (Cai and others 1993; Tiezzi and others 1992) whereas the 90 kD ATP-MAP-labelled organelles follow the MT tracks in the basal part of the tube (Cai and others 2000). The two 400 kD dynein-related polypeptides localized to membrane structures in pollen tubes (Moscatelli and others 1998).

In tobacco, two cDNAs, *TCK1* and *TKRP125,* for kinesin-like proteins have been isolated, but none yet for dynein. *TCK1* originates from a cDNA library from developing anthers of tobacco and is expressed in different tissues of the plant, the highest expression occurring in anthers and stigma (Wang and others 1996). A special feature of TCK1 is a calmodulin-binding domain located in the motor domain at the C-terminus of TCK. Kinesins with comparable structure are also known from potato and *Arabidopsis thaliana* (Reddy and others 1996). The product of bacterially expressed *TCK1* binds calmodulin in a Ca^{2+}-dependent manner. Whether *TCK1* is expressed in pollen tubes has not been reported. *TKRP125* codes for a 125 kD polypeptide isolated from phragmoplasts of tobacco BY-2 cells (Asada and others 1997). The strong similarity of the amino-terminal motor domain of TKRP125 with BimC places TKRP125 in the BimC family with tetrameric kinesin structure (Kim and Endow 2000). The expression of *TKRP125* is cell cycle dependent and its gene product colocalizes with phragmoplast MTs, which suggests the involvement of TKRP125 in cell cycle-dependent changes in arrays of MTs, including organization of the phragmoplast and movement of chromosomes in anaphase (Asada and others 1997). Comparison of the data of *N. tabacum* kinesin cDNAs with the data of kinesin proteins isolated from *N. tabacum* pollen tubes suggests that the different research methods have resulted in identification of different kinesins, which is not surprising because there are at least seven kinesin-related genes in *A. thaliana.*

Research on MT-associated motor molecules may help to reveal the functions of MTs in pollen tubes, which is poorly known compared with the function of MFs. When MTs are depolymerized with oryzalin in *N. tabacum* and *N. alata* pollen grains, germination takes place and the tube elongation without MTs is comparable to that of control tubes (Åström and others 1995), supporting the view that the MFs play a central role during germination and tube elongation. However, in the absence of MTs, the movement of the VN and GC from the grain to the tube tip was significantly delayed and the VN-GC complex was still at the base of the tube when in 90% of control tubes the complex occurred close to the tube tip (Figure 6). The results suggest that MTs are necessary for the fast transport of VN and GC from the grain to the tube tip, and that MT-associated motors might be responsible for rapid movement of the VN and GC complex to the tube tip at the early stage of tube growth. When the oryzalin treatment was continued for 12 h, about 50% of the tubes contained the VN and GC in the middle of the tube or close to the tube tip. All the oryzalin-treated pollen tubes contained MFs, the orientation of which appeared to be similar to those in control tubes (Åström and others 1995). The slow movement of the VN and GC into the tube in the absence of MTs but in the presence of intact MFs could perhaps be due to the function of myosin I localized on the surface of VN and GC (Miller and others 1995). The involvement of the MT cytoskeleton in the movement of the VN and GC from the grain into the tube has also been recorded in germinating pollen of *Endymion nonscriptus* (Heslop-Harrison and Heslop-Harrison 1996), although in this species the time scale of the movement of the VN and GC into the tube is more narrow than in *N. tabacum.*

After the movement of VN and GC to the tube tip, small vacuoles start to appear in the subapical part of the tube in the cytoplasm behind the VN-GC complex, and the first signs of callose plug formation occur. This takes place after about 6 h growth of *N. tabacum* pollen tubes *in vitro.* The formation of callose plugs probably results from local activity of CalS at the plasma membrane and probably requires the transport of CalS in secretion vesicles to the plasma membrane (Figure 7). The accumulation of small vacuoles next to the growing callose plug gradually leads to the division of the pollen tube into the cytoplasm-containing apical part and the empty basal part. This growth pattern appears to be very similar to the *in vitro* growth pattern of the dikaryotic hyphae of the maize pathogenic fungus *Ustilago maydis.*

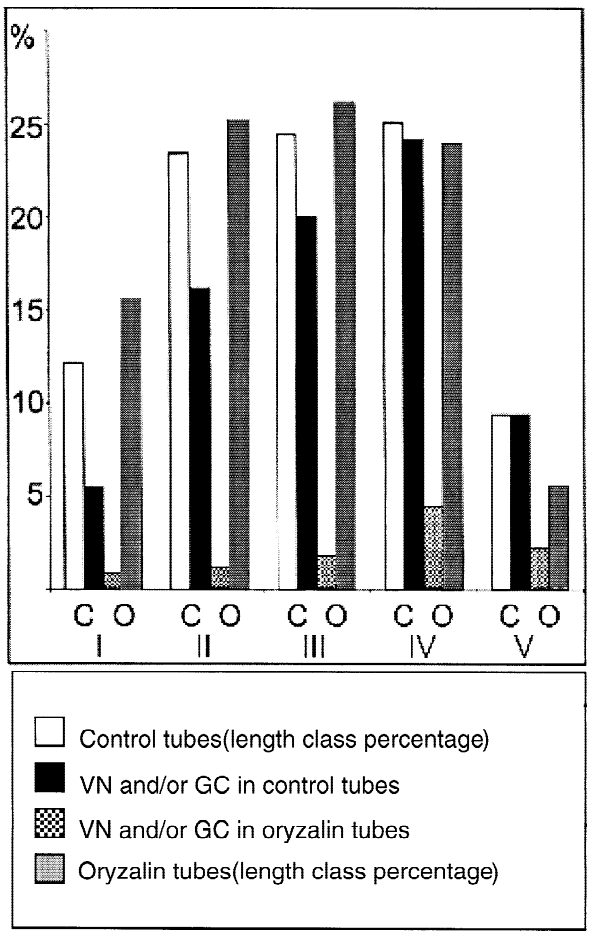

Figure 6. The columns represent the percentages of *Nicotiana tabacum* tube length classes and tubes into which nuclei have moved (VN and GN) on control and oryzalin media. Pollen tubes germinated for 6 h on control (C) or 0.5 µM oryzalin (O) media were divided into the following length classes: 200–300 µm (I), 300–400 µm (II), 400–500 µm (III), 500–600 µm (IV), and 600–700 µm (V). Control tubes n = 479, oryzalin treated tubes n = 478. Note the low percentage of tubes with VN and GC on oryzalin containing media (stippled columns), while the length distribution of the same tubes appears to be comparable to control tubes.

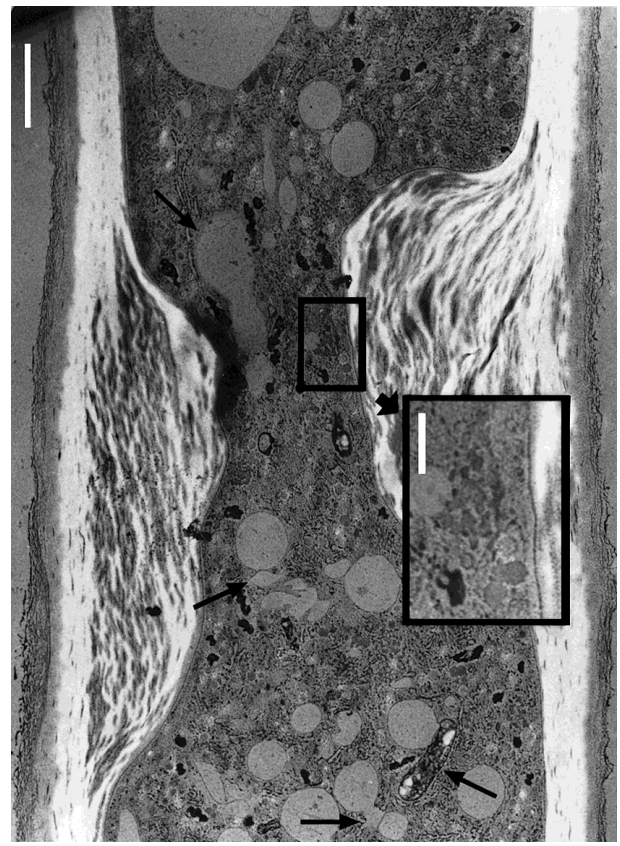

Figure 7. Callose plug in the older part of *Nicotiana tabacum* pollen tube. The cytoplasm contains fusing vacuoles (arrows) and a degrading mitochondrion (arrow). Many Golgi complexes are seen close to the plasma membrane. Boxed area with Golgi complex and secretory vesicles magnified in the insert. Bar 2 µm, bar in insert 1 µm.

In this fungus the formation of empty compartments in the hyphae is dependent on MT-associated Kin2 kinesin motor protein (Steinberg and others 1998). In *kin2* null mutants the amount of vesicles was increased from that in the wild-type hyphae but no basal large vacuoles or empty compartments were observed. These results suggest that *U. maydis* Kin2 MT-dependent motor molecule is involved in vacuolization. The presence of a prominent MT cytoskeleton in the basal part of the pollen tube and similar features in the formation of empty compartments at the base of the cell in pollen tubes and *U. maydis* dikaryotic hyphae warrant attention to the role of MTs and MT-associated motor molecules in the developmental processes leading to vesicle accumulation and vacuolization in the basal part of the pollen tube.

Division of the Generative Cell

In *N. tabacum* the stronger staining by DAPI of GN compared with VN (Figure 4c) and the electron-dense areas of chromatin in GN but not in VN revealed by ultrastructural studies indicate that chromatin is condensed in GC but dispersed in VN. Several unique histone-like proteins (Ueda and Tanaka 1994) and genes encoding them have been isolated from *L. longiflorum* (Ueda and others 2000; Xu and others 1999). The expression of the histone-like genes starts after microspore mitosis and occurs only in the cell that will differentiate into a GC and in the

sperm cells derived from the division of the GC. Immunocytochemistry confirmed that the histone-like proteins accumulate in the elongating and condensing nucleus of the GC during development of binucleate/bicellular pollen. The functional role of these proteins could therefore be to maintain the highly condensed state of chromatin in the GN. They could also mark the progress of GN to the second mitosis necessary for sperm cell development, since the expression of the GC-specific histone-like proteins was not associated with the S-phase of the cell cycle, in which DNA replication and synthesis of histones for interphase chromosome packing are coupled (Ueda and others 2000).

It is generally assumed that the GC in binucleate/bicellular pollen grains progresses through S phase but stops in G2, with the condensed chromatin being a mark of cell cycle arrest, whereas the cell cycle of VN is thought to be arrested already in G1 (McCormick 1993). In comparison, in trinucleate/tricellular pollen grains the presence of two sperm cells in the pollen grain indicates that the cell cycle of the GC is not arrested. Comparisons of cell cycle activities in GCs of binucleate pollen grains with those of trinucleate grains suggest that in binucleate pollen grains the division of the GC could be arrested either by a delay in the synthesis of a compound necessary for progression of the cell cycle to mitosis or perhaps by the presence of an inhibitor of cell cycle progression. Several plant cell cycle proteins, specific CDKs and cyclins, are required for proceeding of the cell cycle from G2 phase to mitosis, but inhibitors of this cell cycle phase have also been identified (Mironov and others 1999). Whether any of these proteins are involved in the GC cell cycle arrest in binucleate pollen remains to be clarified in the future.

The karyo- and cytokinesis of the GC has been frequently a target of intensive research and debate (reviewed by Palevitz and Tiezzi 1992). At the beginning of the nineties the ability to use IIF microscopic techniques renewed interest in the investigation of GC division, particularly in pollen tubes of *Nicotiana* and *Tradescantia* species (Del Casino and others 1992; Palevitz 1990; Palevitz 1993; Palevitz and Cresti 1989; Raudaskoski and others 1987). In *N. tabacum* pollen tubes, metaphase and anaphase configurations with condensed chromosomes as well as two telophase nuclei are observed (Figure 8 a–n) after *in vitro* growth for 17–18 h at 25°C. This indicates that the arrest of GC division is released and division of the GC continues. IIF microscopic observations clearly show that the cage-like structure of MTs in the GC is replaced by a mitotic spindle formed of obliquely oriented MT bundles (Figure 8c). On the basis of these investigations, several groups concluded that the spindle develops from cytoplasmic MT bundles in the GC mainly through reorganization of MT bundles rather than through depolymerization and repolymerization of MTs (Bartalesi and others 1991; Palevitz 1993; Raudaskoski and others 1987), although the disappearance of MT fluorescence from the extensions of the GC on the surface of the VN probably requires depolymerization of MTs. Computer-assisted reconstruction of ultrastructural images from serial sections of GC mitosis in *N. tabacum* (Yu and Russell 1993) excellently describes the reorganization of the MT cytoskeleton during different phases of GC mitosis and explains features that are impossible to interpret on the basis of IIF microscopic studies. The three-dimensional images suggest that after nuclear envelope breakdown the MT bundles could indeed be used directly to form the spindle through capture by the kinetochores of the condensed chromosomes. The analysis of the kinetochore positions in the serial sections indicates that no simple metaphase plate is formed, but that the chromosomes are spread in different planes while attached to the obliquely oriented metaphase spindle. In half-spindles each MT bundle connected to kinetochore at one end is attached by its other end to its own site on ER, lining the lateral sides of the GC. This explains the absence of defined spindle poles in IIF microscopical views (Figure 8c). At anaphase, convergence of multipolar poles of the metaphase spindle to the apical and basal ends of the GC and the simultaneous depolymerization of spindle MTs can be seen as anaphase B-like structures by IIF microscopy (Figure 8e). Smooth ER appears to have a significant role during GC meta- and anaphase (Yu and Russell 1993). ER forms the attachment sites for the multipolar spindle along the lateral sides of the GC, and it appears also to line the spindle MTs, as well as aggregate at the poles of the GC in late anaphase.

No preprophase band (PPB) is observed during the GC division of the pollen tube, which could be an important observation when the phase of the GC cell cycle arrest is considered. During typical (somatic) plant cell division, special CDK-cyclin complexes occur in association with the PPB and are responsible for its disintegration (Colasanti and others 1993; Hush and others 1996; Mews and others 1997). The condensed GC chromatin, the absence of a PPB, and the formation of the spindle without extensive depolymerization of cytoplasmic MT bundles may indicate that the GC is not arrested in G2 but in very early prophase. In this case the MT cage surrounding the GN would be equivalent to the MT organization seen in plant cells just before the

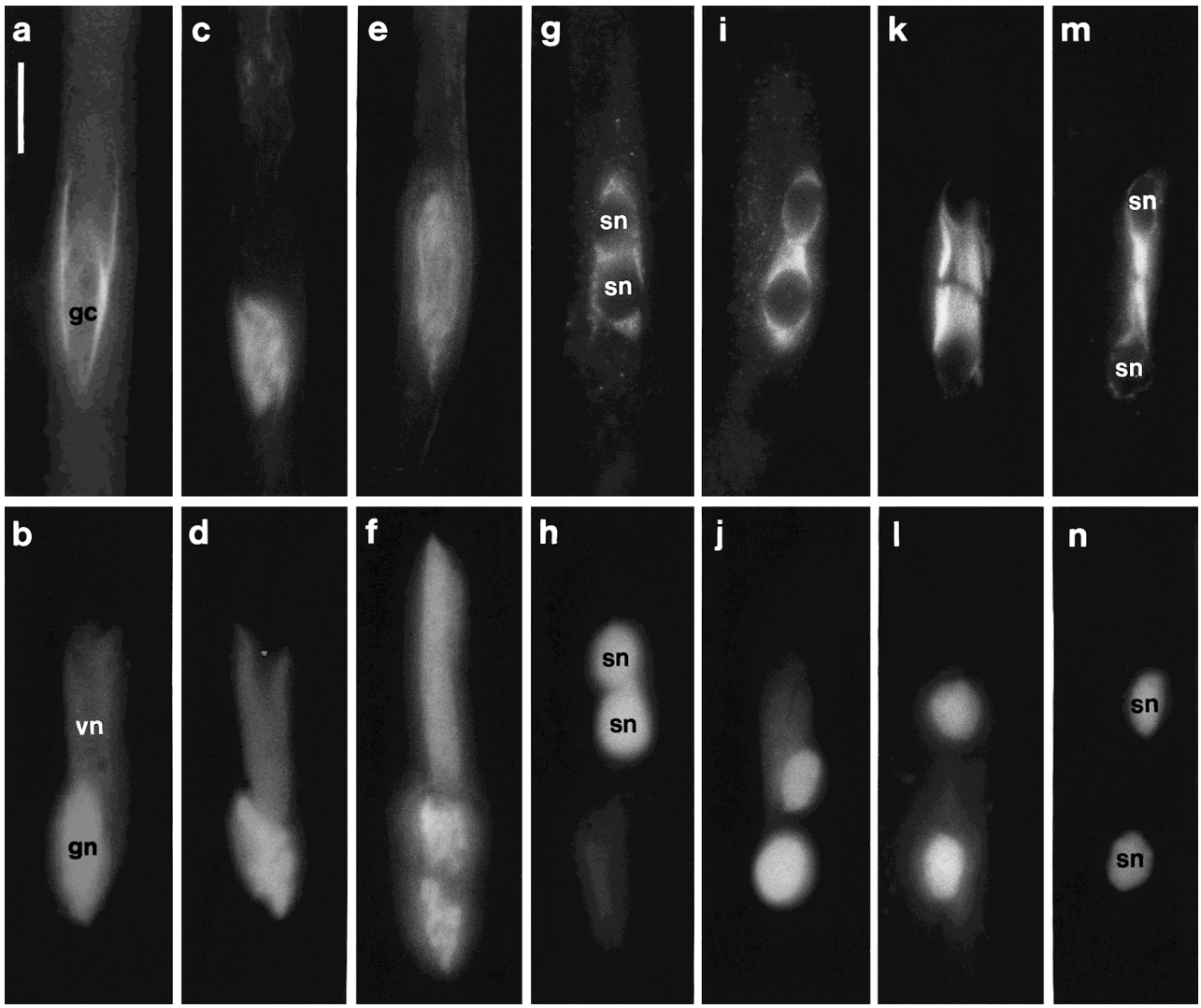

Figure 8. Immunofluorescence with α- tubulin antibodies and DAPI staining of GC division of conventionally fixed **(a–f, k–n)** and freeze substituted **(g–j)** pollen tubes of *Nicotiana tabacum* grown *in vitro* for 18 h. **(a–b)** MT depolymerization and reorganization in GC at the start of the division (a), and DAPI-staining (b) of the nuclei in a. **(c–d)** Oblique mitotic spindle in metaphase. Note the absence of clear spindle poles. Condensed chromosomes of the GN in d. **(e–f)** Spindle elongation at anaphase (e), and the anaphase chromosomes in f. **(g–h)** At telophase, spindle MTs have depolymerized (g) and fluorescence is seen around and between the telophase nuclei (sn) still close to each other in h. **(i–j)** Polymerization of phragmoplast MTs between the telophase nuclei (i), which have moved apart from each other (j). **(k–l)** Typical phragmoplast structure of the GC with the forming cell plate in the middle seen as a gap (k). The telophase nuclei are pushed further apart by the phragmoplast formation (l). **(m–n)** At the end of cytokinesis the phragmoplast narrows and fluorescence in the sperm cells is seen (m). Sperm cell nuclei (sn) in (n) Bar 10 μm.

breakdown of the nuclear envelope. The relationship between the disintegrated nuclear envelope and the specifically oriented ER in GC mitosis is also interesting. The small amount of ER in the GC before division suggests that the remnants of the nuclear envelope instead of ER could perhaps function as attachment sites for the multipolar half spindles along the lateral sides of the GC. MAPs and MT-associated motor molecules responsible for the spindle organization at early metaphase, for the convergence of the subpoles of the half spindles, and for movement of chromosomes at anaphase of the GC division have not yet been identified.

At early telophase, MTs are almost absent from the GC and there is only weak tubulin fluorescence around the two sister nuclei (Figure 8g); this changes to the strong fluorescence of the phragmoplast when MTs are polymerized in the region be-

tween the sister nuclei (Figure 8i). These observations indicate that depolymerization and repolymerization of MTs is a necessary process for GC mitosis and cytokinesis. In the middle of the phragmoplast the newly formed cell plate is seen as a dark gap (Figure 8k) (Palevitz 1993; Raudaskoski and others 1987; Taylor and others 1989), and in ultrastructural studies, Golgi complexes and vesicles associated with MTs are distinguished (Yu and Russell 1993). As in other plant cells, the cell wall separating the sperm cells appears to be built by fusion of vesicles containing cell wall material such as arabinogalactan proteins (Li and others 1995) and perhaps callose (Yu and Russell 1993; Yu and others 1992). After cytokinesis, the length of the sperm cells increases and they become spindle-shaped due to the polymerization of MTs in sperm cell cytoplasm. The distance between the sperm nuclei increases (Figure 8m–n) but the two cells remain together forming the so-called male germ unit (MGU) (Taylor and others 1989; Yu and others 1992). The contents of the two sperm cells in *N. tabacum* and in *Rhododendron* species appear to be similar (Taylor and others 1989; Yu and others 1989), whereas in *Plumbago zeylanica* (Russell 1985), and in some other angiosperms, the sperm cells have an unequal distribution of cell organelles, which might determine their function at fertilization. How the MGU operates *in vivo* at fertilization and how MFs and MTs are involved in this process is one of the most intriguing questions for the future.

Regulation of Cytoskeleton and Pollen Tube Development

The movement of VN and GC from the grain into the tube takes place in *N. tabacum* when the tube has reached a length of 200–300 µm (Åström and others 1995). The signal for the movement could be connected with formation of the tube wall. The expression of CalS starts at germination of the pollen grain (Li and others 1999). At the early phase of germination callose is not present in the tube wall, but it accumulates parallel with tube elongation. This might also involve synthesis and accumulation of other cell wall components. The synthesis of cell wall could lead to the formation of interconnections between a wall component and the plasma membrane, leading to an increase in the translation of cytoskeletal components (Sorri and others 1996) and to the reorganization of cytoskeletal elements in the cytoplasm (Canut and others 1998).

Cytoskeletal changes, involving both MTs and MFs, would then make possible the movement of VN and GC from the grain into the tube tip. *In vivo* the wall maturation in a young pollen tube is probably the prerequisite for the adherence of growth-directing compounds from the stigma and style to the tube (Jauh and Lord 1996; Mollet and others 2000). The two events, the adherence of plant compounds to tube wall and the nuclear movement from the grain to the tube seem not to be dependent on each other, since the VN and GC movement into the tube takes place *in vitro*. However, their simultaneous occurrence is thought to be advantageous for the function of the pollen tube, which is to carry the male gametes in the tube tip to the ovary for fertilization.

The position of the VN and GC close to the tube tip can be thought to divide the function of the tube roughly into two parts; (1) a tip part that contains the secretion machinery and highly efficient exo- and endocytotic pathways for tube primary wall formation and growth and (2) a basal part behind the VN and GC, where the secretion is more directed to secondary wall thickening, callose plug formation, and vacuolar biogenesis. The high 3–10 µM Ca^{2+} concentration in the tip region and the low 0.2–0.3 µM Ca^{2+} concentration in the subapical and basal part of the tube (Franklin-Tong 1999; Franklin-Tong and others 1996; Malhó and Trewavas 1996; Malhó and others 1994; Malhó and others 1995; Messerli and others 1999; Messerli and others 2000; Miller and others 1992; Pierson and others 1994; Pierson and others 1996) must have an impact on the structure and function of the cytoskeleton in these tube regions. In pollen tubes MFs extend closer to the tube tip with the high Ca^{2+} gradient probably due to the presence of numerous ABPs that may modify the structure and function of the actin cytoskeleton in a Ca^{2+}-dependent manner. Only few ABPs have been identified in pollen tubes but all of those appear to be regulated by Ca^{2+}: cofilin activity via phosphorylation by a Ca^{2+}-stimulated kinase (Lopez and others 1996), villin by calmodulin and Ca^{2+} (Yokota and others 2000), and profilin by Ca^{2+} concentration (Staiger 2000).

Recently, the small GTPase Rac/Rop has been shown to play a significant role in pollen tube elongation in pea, *Arabidopsis,* and tobacco (Kost and others 1999; Lin and Yang 1997; Yang and Watson 1993). Both in *Arabidopsis* and *N. tabacum* the small GTPase was found to accumulate at the tube tip. The constitutively active, GTP-bound form led to swollen tip growth whereas the dominant-negative, GDP-bound form led to cessation of tube growth, which proves that the molecule has a central role in the regulation of tube extension. At the pollen tube tip, Rac/Rop seems to activate phosphatidylinositol ki-

nase leading to the formation of phosphatidylinositol 4,5-phosphate, which could function in release of Ca^{2+} from intracellular storages or activate entry from the extracellular space. Transient expression of the mutant GTP- and GDP-bound Rac proteins in tobacco pollen tubes led to formation of extensive and reduced actin cables, respectively, which shows that Rac plays a role in pollen tube actin organization (Kost and others 1999) as in animal cells (Hall 1998).

The stability of the MT cytoskeleton is also dependent on Ca^{2+} concentration in a calmodulin-dependent manner (Bartolo and Carter 1992; Fisher and others 1996). The regulation by Ca^{2+} could explain the absence of MTs from the tip region, the appearance of short MTs in the subapical region, and prominent MT tracks and spirals in the basal region of the tube behind the VN and GC. After conveying the VN and GC from the grain into the tube (Åström and others 1995) MTs probably are involved in keeping the VN and GC complex in the proximity of the tube tip (Heslop-Harrison and Heslop-Harrison 1996). Short MTs in the subapical region in front of and in association with the VN (Raudaskoski and others 1987) are perhaps cooperating with the surrounding MFs, using motor molecules from both cytoskeletal systems as in the bud tip of yeast or at the edge of a migrating fibroblast (Goode and others 2000). This interaction could keep the VN and GC complex near the tip and ensure that the division of the GC also takes place in close proximity to the tip.

The presence of an intact MT and MF cytoskeleton in the basal part of the tube predicts that MTs either directly or in association with MFs are involved in the secretion process leading to tube wall thickening, formation of the callose plugs, and biogenesis of vacuoles, although no data appear to exist yet about the role of cytoskeletal elements in these processes. The formation of vacuoles and empty compartments in the basal part of the pollen tube must be a vital process for the pollen tube elongation *in vivo* and it must also have an effect on tip growth *in vitro.*

In the GC division MTs have, without doubt, an important function, but how GC cell cycle arrest is released and spindle formation is induced from the cytoplasmic MT cage around the GN are not known. During tube elongation the position of the GC close to the tube tip is dependent on the movement of the VN toward the tip, as ascertained by the tight association of the GC to the surface of the VN. The position of the VN-GC complex between the apical and basal secretion poles of the tube could mean that a change on either side of the GC could cause activation of the division. Some circumstantial observations exist, mainly from the investigations of *Nicotiana* pollen tubes, about factors that seem to affect the GC division. The time of GC division may be shortened by growth medium composition and temperature (Read and others 1993); nanomolar concentrations of the serine/threonine phosphatase inhibitor okadaic acid inhibit the GC division when applied at the right time to the elongating pollen tube (Raudaskoski and others 1997); and in 24 h-old pollen tubes the levels of tubulin and actin are lower than at an earlier stage of growth (Sorri and others 1996). In addition, at the time of GC division, alterations in tube tip structure must occur because the tip bursts extremely easily in response to any environmental change. These observations suggest that the release of GC cell cycle arrest or the induction of GC division reflects some metabolic alterations in the pollen tube cytoplasm. Perhaps there is a decrease in the amount and stability of mRNAs and proteins transcribed and expressed in the early stage of germination (Bate and Twell 1998), which induces a signal for a phosphorylation and dephosphorylation cascade that leads to the release of GC cell cycle arrest.

Future Aspects

This review deals only with a small portion of the data available on pollen tube growth and development, most of which has been published after IIF microscopical research revealed the extensive cytoskeleton in pollen tubes. For the most part, the literature deals with *in vitro* experiments, because much less data are available from *in vivo* research. During the last five years or so rapid development of techniques for Ca^{2+} imaging together with interest in the role of Ca^{2+} in regulating cytoskeletal organization and secretion has enormously increased the amount of research on pollen tube tip growth. Investigations resolving how the *in vitro* results fit with the role of Ca^{2+} in *in vivo* tube growth will certainly follow (Messerli and others 2000). Recently it was shown that in pollen tubes of *Papaver rhoeas* the actin cytoskeleton is a target for signal transduction associated with the gametophytic incompatibility response (Geitmann and others 2000). This observation may draw attention to the possibility of using pollen tube cytoskeletal organization as an indicator of downstream events in signal transduction pathways in other gametophytic and in sporophytic incompatibility systems.

The observation that several compounds produced in the pistil affect pollen tube growth will also increase the interest in factors regulating *in vivo*

growth of pollen tubes. Methods have already been developed by which the compound produced *in vivo* is used *in vitro* to reveal how products from the style regulate growth of the pollen tube (Cheung 1996; Lord 2000). In the future this research probably needs to take into account the effect of stigma- and style-secreted compounds on cytoskeletal organization during elongation of the pollen tube *in vivo*.

The use of pollen tubes in transgenic expression of GFP-labelled cytoskeletal components (Kost and others 1998) made it possible to follow the behavior of the cytoskeleton in living tubes, observations also made possible by microinjecting cytoskeletal proteins tagged by fluorochromes into pollen tubes (Miller and others 1996; Vidali and Hepler 1997). By using these techniques, a more accurate understanding can be gained of the specific role of MTs and MFs in VN and GC movements in the pollen tube and of the alterations in the MT cytoskeleton at GC division. Expression of a gene of interest under the control of a pollen tube specific promotor (Bate and Twell 1998) has unexpectedly proved that the small GTPase protein Rac has a significant role in regulating pollen tube tip growth (Kost and others 1999; Zheng and Yang 2000). These recent experiments and results are only the beginning of research on the structure and function of the pollen tube cytoskeleton.

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