

REVIEW

Regulation of pollen tube growth

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Key-words: pollen tubes, tip growth, cell–cell interaction, cytoskeleton, cell wall, endocytosis, exocytosis, calcium.

INTRODUCTION

Most textbooks describe pollen and pollen tubes as the vectors carrying the male sperm cells in seed plants. The pollen tubes grow through the style to the ovule, where the sperm cells are delivered to the egg cells in the embryo sacs. Pollen tubes always grow in an alien environment: in evolutionary terms they derive from the haustorium through which the primitive microgametophyte fed on the sporophyte. Haustorial forms are still found in the case of ramified pollen tubes of gymnosperms that survive and feed for a long period in the female cone.

Pollen tubes have a specific form of intrusive growth, or tip growth. Other cells with tip growth include root hairs, bipolar extending tracheids and fibre cells, and also many organisms such as a number of algae, moss and fern protonemal cells and most fungal hyphae. Diverse as they are, these cell types basically share the same cytoplasmic constituents and thus may show many similarities in behaviour to each other. However,

Abbreviations: AGP, arabinogalactan-proteins; AF, actin filaments; CF-FS, cryo-fixation—freeze-substitution; CLSM, confocal laser scanning microscopy; CMF, cellulose microfibrils; CP, coated pits; CV, coated vesicles; EGTA, ethylene glycol-bis (β amino-ethyl ether) N,N,N',N'-tetra-acetic acid; ER, endoplasmic reticulum; HRGP, hydroxy-proline rich glycoprotein; MAP, microtubule associated protein; MT, microtubules; MVB, multivesicular body; PEG, poly ethylene-glycol; SV, secretory vesicles; TGN, trans- Golgi network; UV, ultra-violet; VEC-DIC, video enhanced contrast-differential interference contrast.

the large evolutionary distance between the fungi (including Oomyceta) and the Phaeophyta may have allowed different modes of tip growth to develop.

Pollen tube growth is often not uniform; large differences occur *in vivo* as well as *in vitro*. Branching or production of more than one tube per grain often occurs. Both directional and non-directional growth have been observed.

Pollen tubes are important as the independent microgametophytic part of the plant's life cycle. Their role in gene transfer, fertilization and propagation of seed plants and their use as a model system to study plant cell growth and cell-cell interactions ensure that pollen tubes are studied as an important aspect of plant science. Basic aspects of tube physiology and cytology were first described about 50 years ago. An excellent review of these pioneering studies is that by Maheswari (1949). A comprehensive study of the physiology and cell biology of pollen tubes has been given by Iwanami (1959). Later reviews on pollen tube growth and physiology are those by, for example, Knox (1984), Linskens (1967), Linskens & Kroh, (1970), Heslop-Harrison (1987) and van Went & Willemse (1984); for Gymnosperms see Singh (1978). Tip growth has been reviewed by Sievers & Schnepf (1981). Recent reviews on various aspects of pollen tube growth are those by Steer & Steer (1989) and Mascarenhas (1993). Here we will focus on the relationship to growth regulation of cytoplasmic organization, cell wall, cytoskeleton, exo- and endocytosis and calcium.

POLLEN TUBE GROWTH

Direct demonstration of tip growth is difficult to achieve in any tip growing system (Derksen & Emons 1990). Tip growth in pollen tubes was first recognized in *Veronica* (Schoch-Bodmer 1932). Experimentally it has only been demonstrated in pollen tubes of *Lilium* (Rosen 1964). However, in most types of pollen tubes studied so far, patterns of small irregularities on the surface behind the tip remain unaltered during tube growth, thus proving that cell wall extension does not occur in the main tube. In lily, stretching of the primary wall has been estimated to occur up to a distance of 5 μm behind the very tip (Rosen 1964).

Rosen (1971) suggested two periods of growth for lily pollen tubes: a period of autonomous growth, sustained by pollen resources, followed by a period of heterotrophic growth, sustained by imports from the style. According to Mulcahy & Mulcahy (1983) the heterotrophic period is marked by the appearance of callose plugs in the wall and the occurrence of the second mitosis. This was supposed to be the case in bi-nucleate pollen that initially grows slowly, but rapidly increases growth rate afterwards.

Autonomous growth shows the principal ability of the pollen tube to grow without external resources. Autonomous growth is not believed to occur in trinucleate pollen whose sperm cells need not undergo a further mitosis, are poor in polysome content and various metabolites, and can immediately form callose plugs (see also Heslop-Harrison 1987). Such pollen is noted for the difficulty involved in germinating and growing it *in vitro*. Autonomous or heterotrophic growth may be highly variable as shown for the different sensitivities of various species to cycloheximide and actinomycin D (Knox 1984; Mascarenhas 1993). There is no evidence for a specific mRNA synthesis during pollen tube growth (Mascarenhas 1993). However, the observations clearly demonstrate the importance of considering the biology of the pollen tube in any experimental

approach: most experimentation is carried out on heterotrophically growing binucleate pollen tubes, without information on expression and turn-over of relevant proteins or mRNAs.

A variety of media are described for *in vitro* pollen tube growth, ranging from simple sucrose/boric acid media (Linskens 1967) to highly complex media containing poly ethylene-glycol (PEG) (Zhang & Croes 1982) and many amino acids (e.g. Read *et al.* 1993). Pollen of *Pinus* will even germinate and grow on plain water (for survey of various media, see Stanley & Linskens 1974). However, a high concentration of calcium is always required, either from internal (including the pollen coat) or from external sources (Brewbaker & Kwack 1963). The other requirements for *in vitro* growth are highly variable and besides nutritional compounds, various ions, including potassium and hydrogen ion level (pH), O₂ pressure and osmolarity (including the nature of the osmoticum) and viscosity of the media may be important. Indeed, incongruity, that is the incapacity of pollen to germinate on stigmas of other species, often may depend on deficiencies in these conditions at the stylar surface or even in the styles. Hormones may affect pollen tube growth, but their effects are highly variable in different species for different hormones. They may stimulate or retard growth, cause branching, show no effect, etc. No consistent pattern is apparent (e.g. Iwanami 1959; Sondheimer & Linskens 1974; Stanley & Linskens 1974).

Pollen tube growth *in vivo* may be quite different from the uniform growth seen in most *in vitro* systems. For example, initially multisiphonous tubes occur in *Impatiens*, *Oenothera* (Iwanami 1959), *Malvaceae*, *Cucurbitaceae* and *Campanulaceae*, whereas ramifications or bifurcations near or even in the nucellus have been shown to occur in *Carpinus*, *Fagus*, *Ulnus*, *Myosurus*, *Iris*, *Peperonia*, *Portulaca*, *Phryma* and also in *Petunia* (Maheswari 1949). The last, *Petunia*, is often used in *in vitro* studies. In the genus *Clarkia*, and in other members of the Onagraceae, the occurrence of initially multiphosphonous pollen and branching pollen tubes has been related to lack of contact to the stigma, due to clumping of pollen tubes (Smith-huerta 1991). *Spinacia* pollen tubes grow evenly, but after fertilization they ramify into haustoria-like structures, supposedly as a result of a factor released by the fertilized ovules (Wilms 1974). *Prunus* pollen tubes lose directional growth and start curling and branching in ovaries with non-viable ovules, supposedly due to a lack of suitable attractants (Arbeloa 1994). Similar observations, i.e. reversal of growth orientation have also been made on *Brassica* ovules that cannot be fertilized (Mitterpergher *et al.* 1994). In *Oenothera*, fertilization occurs only in 'genetically correct' ovaries. Pollen tubes grow in an even fashion into the style, but only in genetically correct ovaries does growth become directed towards the ovules, where the tubes branch before fertilization (Schwemmler 1968; Glenk 1964). Such observations show that there are numerous factors dictating the behaviour of pollen tubes, so that there are no general patterns of behaviour that apply to all species.

There are many examples of directional pollen tube growth, both *in vitro* and *in vivo*, in response to specific tropic agents from stylar exudates, style tissues and ovules. Besides specific agents, nutrients and inorganic ions, especially calcium, have been reported to cause tropic reactions (see Linskens 1964; Van Went & Willemse 1984). However, early studies on germinating pollen populations may not always have discriminated between growth stimulation of the pollen tubes at the site of the highest concentration and true chemotropism seen as a change in orientation caused by the substance or tissue in question (Heslop-Harrison 1987). The role of chemotropism in

pollen tube growth *in vivo* has been critically discussed by Heslop-Harrison & Heslop-Harrison (1986). True chemotropism may occur in lily, for example (Iwanami 1959; Rosen 1964), *Anthirrinum* (Mascarenhas & Machlis 1964), *Oenothera* (Glenk 1964), *Penisetum* (Reger *et al.* 1992) and *Gasteria* (Willemse *et al.* 1994). The simple fact that pollen tubes find their way into the style, even in species without mechanical guiding systems, and follow specific routes to the ovary, inescapably argues for the presence of chemotropic agents. It may be that directionality of growth is not an issue during stylar growth, but is important on the stigma, when the pollen tubes enter the sporophyte, and at the ovary when they have to enter the female gametophyte. The synergids have been reported to extrude Ca^{2+} before pollen tube entry, which may act as a chemotropic agent (Chaubal & Reger 1993 and refs therein; for discussion on older literature: see Van Went & Willemse 1984; see also Mascarenhas 1993).

CYTOPLASMIC ORGANIZATION

The cytoplasm of the pollen tubes shows a particular organization that is thought to be related to, or even a prerequisite for, tip growth. Occurrence and function of the various organelles have been extensively discussed by Steer & Steer (1989) and Morr  (1990). Organelle movement has been reviewed by Emons *et al.* (1991).

Sassen (1964) and Rosen and co-workers (1965) were the first to show the significant accumulation of secretory vesicles (SV) in the tip at the site of exocytosis (Fig. 1A). This accumulation is probably identical to the so-called 'hyaline zone' or 'cap block' seen in many species by Iwanami (1959). Also, other organelles such as mitochondria, dictyosomes and endoplasmic reticulum (ER) have been reported to be more abundant in a broad zone behind the growing tips of a variety of pollen tubes (Cresti *et al.* 1977, 1985; Miki-Hirosige & Nakamura 1982; Reiss & Herth 1979; Reiss & McConchie 1988; Uwate & Lin 1980; Steer & Steer 1989). Recently, Derksen *et al.* (1995) used cryo-fixed and freeze substituted (CF-FS) pollen tubes of tobacco (see also Fig. 1) to quantitatively study organelle distribution. An uneven distribution was recorded for SV and dictyosomes, but they could not confirm accumulations of mitochondria. Though they could not quantify the ER, smooth tubular ER was clearly highly abundant near the growing tip whereas cisternal, rough ER was not. In cryo-fixed lily pollen tubes, ER was present in between the SV (Lancelle & Hepler 1992), but in tobacco it was not (Derksen *et al.* 1995). Whether the differences between these reports and earlier studies are due to a redistribution of organelles caused by the use of aldehyde fixatives, similar to those reported for the hyphae of the fungus *Saprolegnia* (Kaminskyj *et al.* 1992), or whether they result from the use of different species and different culture conditions, is not known. In *Tradescantia*, strong fluctuations in zonation may occur (Steer & Steer 1989).

Derksen and co-workers (1995) also observed a steep gradient in the distribution of the coated pits (CP) that represent an endocytotic route in plants (Tanchak *et al.* 1984). Such a marked distribution clearly points towards a specific function related to pollen tube growth.

A different cytoplasmic organization may occur in gymnosperm pollen tubes. *Pinus* pollen initially forms typical tubes, but they branch markedly after prolonged culturing. In the tips of growing tubes, including the branches, there is an accumulation of SV though less prominent than in the angiosperm pollen tubes. The cytoplasmic organization behind the tip is different. Mitochondria and dictyosomes are more often found near the wall, indicating a radial zonation, whereas a longitudinal zonation seems to be

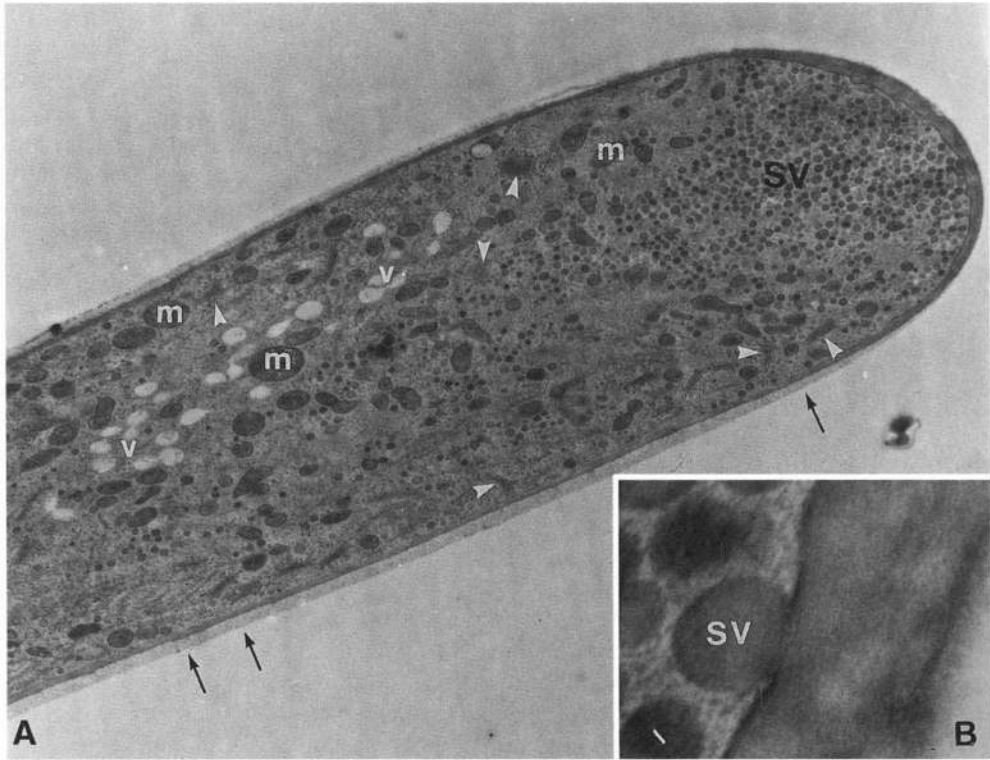


Fig. 1. (A) Median longitudinal section through the tip of a freeze-fixed and freeze substituted pollen tube of *Nicotiana tabacum* cultured *in vitro* in a sucrose borate medium (Derksen *et al.* 1995). In the tip the zone with secretory vesicles is indicated (SV). Note the thick wall in the tip and the less electron dense outer layer (arrow) in the tube that gradually emerges from the wall secreted in the tip. Mitochondria, m; vacuoles, v; dictyosomes, arrowheads. Magnification: $\times 20\ 000$. (B) Secretory vesicle fused with the wall. Note the similar electron density of the vesicle and the primary wall in the tip. The outermost part of the wall has a 'fluffy' appearance. Magnification: $\times 280\ 000$.

absent (A. H. N. de Win & J. Derksen, unpublished data). As has been argued before (Herth *et al.* 1990), zonation is not necessarily static, but may result from organelles moving into and out of the tip. The typical zonation may not be absolutely required for tip growth, but may be related to increased growth rates (see also Sievers & Schnepf 1981).

All angiosperm pollen tubes investigated so far show the typical reverse fountain-like streaming of the cytoplasm originally described by Iwanami (1959) for lily. However, after prolonged culturing the large vacuole, originating from the grain, moves towards the tip and the streaming pattern may change from reverse fountain-like to a rotating or circulating pattern (lily, Iwanami 1959; tobacco, De Win & Derksen, unpublished data). Detailed studies using video-enhanced contrast, differential interference contrast (VEC-DIC) and fluorescent probes for mitochondria show that organelles move independently of the stream. Organelles move at highly variable speeds. They may move at a constant speed over a long distance, and then the motion may be interrupted or even become reversed (Herth 1989; Pierson *et al.* 1990; Emons *et al.* 1991). However, movement of organelles may be linked to each other, as mitochondria have been shown to move along smooth tubular ER (Pierson *et al.* 1990), and bridges between

mitochondria and ER tubules are seen in electron micrographs. Associations of ER with other organelles, such as vacuoles, were also observed (Derksen *et al.* 1995 and refs therein). Due to the lack of specific markers, the movement of organelles other than mitochondria is less well described. Movement of organelles within the tip is likely to be linked to tip growth mechanisms. Initial observations indicate lower average speeds with more non-directional movements near the tip as compared to the tube (De Win & Derksen 1994), thus accumulations of some organelles at the tip might reflect a different type of motion in this region. No differences in ultrastructure were observed between organelles at the tip and in other parts of the pollen tube (Derksen *et al.* 1995).

CELL WALL

The structure and composition of the wall have been comprehensively discussed by Heslop-Harrison (1987) and Steer & Steer (1989). By definition, the wall at the tip is a primary wall. However, a peculiarity of pollen tube growth is differentiation into a growing tip and a non-growing tube, so wall formation in the non-expanding tube has sometimes been considered to be secondary (Kroh & Knuiman 1982). The wall formed at the tip is continuous over the tip and around the tube. It is composed chiefly of pectins/hemicelluloses and possibly cellulose. Cellulose microfibrils (CMF) form the internal pressure-resistant framework of the plant cell (Cosgrove 1993). In early studies they were reported to occur in both the tip and tube wall in *Petunia* (Sassen 1964) and *Lilium* (Dashek 1966). They were found in preferential orientations of about 45° to the long axis in the tube, whereas in the tip they did not show preferential orientations. Such images are obtained after vigorous extractions that only leave ghosts with the crystalline cellulosic part of the wall (Engels 1974a,b; see also Kroh & Knuiman 1982). However, the nature of the fibrils in these ghosts has been questioned (Steer & Steer 1989) as has their occurrence and density in the tip and the secondary wall (Linskens 1967; Steer & Steer 1989).

Recently, networks similar to those observed in *Petunia* were found in the primary and secondary walls of tobacco (Van Amstel 1994) and *Pinus* pollen tubes (Derksen & De Win, unpublished data). For tobacco pollen tubes grown *in vitro* it could be shown that the ghosts contained true crystalline cellulose (β ,1-4 D glucan) as they displayed the typical cellulose X-ray diffraction pattern (A. Van Amstel, unpublished, but see Herth *et al.* 1974 for a critical assessment of such patterns). The deposition of the cellulose microfibrils may be similar to that of other primary walls (Wolters-Arts *et al.* 1993). The CMF network formed on protoplasts from pollen tubes is not capable of maintaining the globular cell form (Rutten *et al.* 1991) and as the *in vitro* tip is thin and the tube is not supported by surrounding tissues, the possible role of CMF in resisting turgor pressure should not be over-emphasized (see also below and cytoskeleton).

The wall covering tip and tube appears fibrous, striated and electron dense in ultrathin sections in the electron microscope. Sometimes a differentiation into a pectic outer layer and a cellulosic inner layer was observed. In the tip the cellulosic layer sometimes appeared thinner or absent (Heslop-Harrison 1987; Steer & Steer 1989). In CF-FS pollen tubes of lily (Lancelle & Hepler 1992) and tobacco (Derksen *et al.* 1995) the wall at the tip appears very thick and rather homogenous (Fig. 1). The wall had the same high electron density as the wall material in the secretory vesicles of the tip and did not show any striations or a differentiation into a pectic and cellulosic layer (Derksen *et al.* 1995). Therefore, it was concluded that this wall had to be highly fluid and would

gradually separate into the electron translucent outer (pectic) layer and a striated, inner (cellulosic) layer. The inner layer could equally be formed by synthesis at the plasma membrane of the main tube behind the tip. As the outer layer was seen to easily dissolve into the medium (Derksen *et al.* 1995; Van Amstel 1994), secretion via the wall of the tip may be the route by which functional active substances reach the environment. Perhaps the proteins described by Čapková (1994) are dispersed into the medium via this route. Fungi similarly secrete proteins via the hyphal tip only (Wessels 1993).

The relationship between turgor and expansion is far more complicated than generally understood. As already noted by Zeigenspeck (1920), expansion is more determined by wall synthesis than by turgor pressure. Recently, Rutten & Knuiman (1993) used Brefeldin A, an inhibitor of the anterograde membrane transport, to specifically inhibit SV formation. This resulted in continued growth till all SV were used up and growth stopped. These results clearly show exocytosis as one of the factors determining tube growth.

Recently, Li and co-workers (1994) observed that pectins in the tip were esterified, whereas in the tube they were acidic. Esterified pectins do not mutually interact and give a plastic character to the wall, whereas acidic pectins strongly interact in the presence of Ca^{2+} , as shown for tobacco pollen tubes (Tirlapur *et al.* 1994) and give rise to a relatively inflexible and rigid wall. These observations not only provide further evidence for the plastic character of the wall in the tip, they also show that high Ca^{2+} concentrations may not greatly affect its rigidity. The pectins are probably secreted in a methyl-esterified form (Tirlapur *et al.* 1994). Since secretory activity is restricted to the very tip and, probably, only a single type of SV exists in tobacco (see *Exocytosis*), de-esterification must occur in the tip by an enzyme simultaneously secreted with the pectins. Continuous secretion then would lead to a gradient from esterified to de-esterified pectins in the dome accompanied by a progressive loss of plasticity. Expansion of the wall in the tip is not simply surface enlargement as if one were blowing up a balloon, but must follow a distinct pattern governed by tip to base changes in the elastic and plastic properties of the wall, the force being determined by the tube's turgescence. The present observations on cellulose and pectins precisely answer the requirement of a distinct pattern in wall extension. At the cellular level, a similar behaviour is also expected to occur in the tips of fungal hyphae (Wessels 1988, 1993). Obviously, conditions that favour esterification or de-esterification would strongly affect pollen tube growth.

Pollen tube walls also contain hydroxy-proline rich proteins (Dashek & Harwood 1971; Li *et al.* 1983). Inhibition of proline hydroxylation seems to affect lily pollen tube growth (Dashek & Harwood 1971, 1976). Arabinogalactan-proteins (AGP) may be present (Li *et al.* 1992, see below), but their relation to the previously described hydroxy-proline rich proteins remains unclear. These proteins belong to the group of hydroxy-proline-rich glycoproteins (HRGP; review: Showalter 1993; Kielszewski & Lampert 1994). Which HRGP is present and what function they might have is completely unknown. However, they might connect the plasma membrane to the cell wall in a similar way to the sites cross-reacting with anti-HRGP antibodies at the Hechtian attachment sites as proposed by Pont-Lezica *et al.* (1993).

In the tube a secondary wall is formed that is mainly of a callosic (β ,1-3 glucan) nature, though many other, often poorly characterized components occur as well. In the electron microscope this wall is seen as an amorphous, electron translucent layer with many inclusions (Heslop-Harrison 1987; Steer & Steer 1989). CMF were also found in the secondary wall of tobacco (Van Amstel 1994). The cellulosic and callosic

components of these walls are synthesized at the external membrane surface by transmembrane complexes that supposedly arrive via SV. Putative cellulose synthesizing complexes have been observed on SV in plant cells (Herth 1985) and in lily pollen tubes they have been shown to occur at the plasma membrane (Reiss *et al.* 1985).

Cellulose synthases are believed to have a short life-time and then convert to longer lasting callose synthases (e.g. Northcote 1991). In contrast to ordinary cells, callose synthases of pollen tubes are not Ca^{2+} -sensitive (Schlöpmann *et al.* 1993) and therefore may not be related to cellulose synthases. Perhaps the hexagonal arrays in the membranes of tobacco pollen tubes (Kroh & Knuiman 1985a) represent callose synthases. Fluorescence imaging with calcofluor white, a fluorescent dye, that preferentially binds to cellulose, and autoradiography of ^3H -UDP-glucose labelled and extracted pollen tubes, together with observations on extracted walls in the electron microscope showed that the very thin CMF network gradually thickened towards the base of the tube (Van Amstel 1994), thus indicating a continuous cellulose synthesis. The occurrence of inclusions of various chemical compositions indicates that exocytosis into the secondary wall must occur, though perhaps very rarely.

The occurrence of callose is to be interpreted as a barrier separating the cell from its environment, as for example in microsporogenesis (Heslop-Harrison & Mackenzie 1967; Rodriguez-Garcia & Majewska-Sawka 1992). Callose linings occur often between cells that are in close contact, especially if they do not share an identical background (Gorska-Bryllass 1986). With maybe a few exceptions, i.e. *Cosmos bipennatus* (Knox 1973) and *Lychnis alba* (Crang & Hein 1970), all pollen tubes have callose linings. Therefore, it must be expected that generally cell-cell interactions may only take place where callose is almost absent, that is near to the tip, as shown for tobacco and lily pollen tubes (Rae *et al.* 1985; Van Amstel 1994). In the electron microscope the thickness of the secondary wall can be seen to gradually increase towards the base, as was also shown for *Petunia* (Herrero & Dickenson 1981). Staining with aniline blue, a dye specific for callose (Linskens & Esser 1957) sometimes reveals a banded pattern in both tobacco and lily (Van Amstel 1994). It is not clear whether this pattern reflects local differences in callose content or is caused by local differences in the complex structure of the callose (on callose see Evans *et al.* 1984).

Using specific antibodies Li and co-workers (1992, 1994) showed bands of arabinogalactan proteins and acid pectins in the primary wall of tobacco pollen tubes. As for callose, the origin of the patterns is unknown and a correlation between the various banding patterns has not yet been made. A possible correlation might be suggested with pulsating growth as seen in *Gasteria* pollen tubes (Willemse *et al.* 1994): banding might be brought about by alternating periods of wall thickening and rapid tip expansion (Pierson *et al.* 1995).

CYTOSKELETON

The cytoskeleton of plant cells consists of microtubules (MT) and actin filaments (AF) that are seen as microfilaments in the electron microscope. Though proteins that share epitopes with the intermediate filaments of animal cells probably are present in plant cytoskeletons, intermediate filaments seem to be absent (review: e.g. Derksen *et al.* 1990).

Recent reviews dealing with various aspects of the cytoskeleton in pollen tubes are those by Derksen & Emons (1990), Pierson & Cresti (1992), Steer & Steer (1989) and

Steer (1990). MT and AF were first observed by electron microscopy in the seventies (Franke *et al.* 1972) and AF were identified by heavy meromyosin decoration (Condeelis 1974). Due to their sensitivity to chemical fixatives they are frequently lost during specimen preparation (Derksen & Emons 1990), so that their presence and function, especially of MT, were questioned (Heslop-Harrison 1987). The advent of fluorescent probes for cytoskeletal proteins has greatly facilitated cytoskeleton studies in whole pollen tubes (Fig. 2). MTs were first visualized by means of immunological probes in *Nicotiana* pollen tubes (Derksen *et al.* 1985; Raudaskoski *et al.* 1987). Later MTs were seen to be present in all species investigated (Derksen & Emons 1990; Pierson & Cresti 1992). Actin filaments were mainly detected with the fluorescent probe rhodamine-phalloidin (Parthasarathy *et al.* 1985; Perdue & Parthasarathy 1985; Pierson *et al.* 1986), but also with immunological probes (Pierson *et al.* 1986). In tobacco and lily pollen tubes no significant differences were found between rhodamine-phalloidin stained tubes and antibody labelled tubes (Pierson *et al.* 1986), but different staining patterns have been observed in cytochalasin treated tubes (Tang *et al.* 1989b). Rhodamine-phalloidin was initially used in combination with formaldehyde as a fixative, but later it appeared more effective when combined with extraction procedures under AF stabilizing conditions (Pierson 1988). MT and AF are readily preserved by cryofixation and freeze substitution, allowing study of their distribution in thin sections (e.g. Lancelle *et al.* 1987). Plasma membrane-bound MT and AF can be studied in relation to other membrane bound structures like coated pits by means of the dry-cleaving technique (Derksen *et al.* 1985). Visualization techniques for pollen tube cytoskeletons have been reviewed by Derksen & Emons (1990) and Pierson & Cresti (1992).

In pollen tubes, microtubules are mainly cortical in their distribution. Fluorescent probes show arrays of mainly axial or sometimes helical orientations, but near to the tip they become less organized and seem to become more randomly distributed. (Derksen *et al.* 1985; Pierson *et al.* 1986; Derksen & Emons 1990; Cresti 1992). In *Nicotiana* (Del Casino *et al.* 1993) and *Zea* (in Pierson & Cresti 1992) circumferential MT have been observed behind the tip, but this is not a common arrangement. In these preparations MT easily break, giving rise to interrupted lines of fluorescence. Tyrosinated and acetylated tubulins have been reported to occur in pollen tubes (Åström 1992; Del Casino *et al.* 1993).

MT are often reported to be absent from the pollen tube tip. In *Nicotiana* pollen tubes microtubules were shown to be present in criss-cross patterns (Fig. 2A) in the tip using confocal laser scanning microscopy (CLSM) (see also Cai *et al.* 1993). Sometimes these MT even traverse the tube. They may not have been recorded earlier due to the geometry of the tip which could blur signals from the internal cytoplasm. The presence of short randomly distributed microtubules in the tip means that they are rarely detected in thin sections (Fig. 2A; see also Lancelle *et al.* 1987). Åström and co-workers (1991) showed MT near the tip of tobacco pollen tubes were sensitive to cold shock. During early stages of recovery, MT reformed in random orientations. These events resemble the sequence of MT polymerization seen in platelets after MT depolymerization by a cold shock (Behnke 1969).

Pollen tube growth is relatively insensitive to MT depolymerizing drugs like colchicine, so it was assumed that they did not play a significant role in pollen tube growth (Franke *et al.* 1972). Recently, Joos and co-workers (1994) reported a relatively high sensitivity to colchicine of older, highly vacuolized pollen tubes of *Nicotiana*. They also observed changes in cytoplasmic organization, i.e. displacement of the vacuoles.

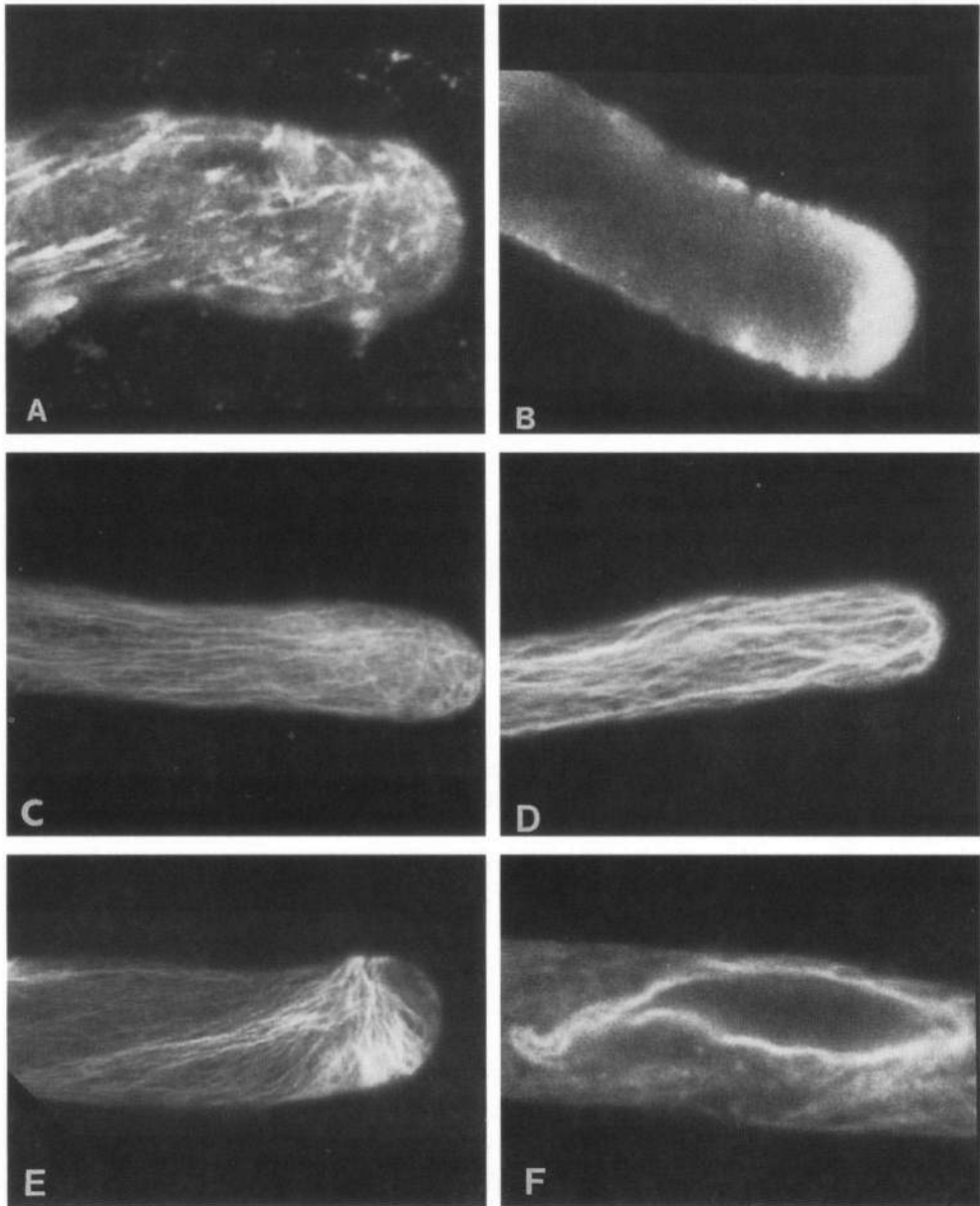


Fig. 2. Immunofluorescence staining of (A) tubulin (essentially according to Derksen *et al.* 1985); (B) a putative spectrin-like protein, and (C–F) F-actin. In (B) the primary antibody was a rabbit anti-chicken spectrin (Sigma S-1390). The second antibody was a FITC labelled goat anti-rabbit. In (C–F) TRITC-phalloidin labelled F-actin in permeabilized pollen tubes was used (after Pierson *et al.* 1986). All images were made in the confocal laser scanning microscope. (C) The normal F-actin pattern. The thick bundles in the tip (D) and the circular configurations (E) are caused by 1 μm EGTA treatment, they are non-growing tubes. In (F) the characteristic configuration of F-actin around the generative cell is shown. Optical sections (3–4) taken at intervals of 0.5 μm were combined, except (F) which represents a single optical section. Magnifications: (A), (B), (F): $\times 5000$; (C), (D), (E): $\times 4000$.

These data seem to contradict earlier observations on both *Nicotiana* and other species, where no or only little effect was seen (e.g. Franke *et al.* 1972; Derksen & Traas 1984; see also Steer & Steer 1989). However, most of these reports concerned pollen tubes grown only for a few hours *in vitro*. Perhaps the difference can be explained by a more prominent anchor function of the MT in older pollen tubes (see below). The MT observed patterns do not match the CMF patterns in the wall, so a controlling function of cortical MT in this respect must be rejected (Emons *et al.* 1992). Connections between MT and a number of organelles have been reported (for survey see Pierson & Cresti 1992), but their functions are unknown. In plant cells (e.g. Kengen & Derksen 1991), including pollen tubes, MT occur in bundles (Franke *et al.* 1972; Pierson *et al.* 1986). Bundling obviously strengthens and stiffens the microtubular skeleton, which may point towards a true skeleton or anchor function (see also below).

Only recently have microtubule-associated proteins (MAP) been investigated in pollen tubes and other plant cells. A kinesin-like protein with MT-dependent ATP-ase activity has been isolated from *Nicotiana* pollen and localized on organelles, probably dictyosomes and related vesicles, and the plasma membrane in pollen tubes (Tiezzi *et al.* 1992; Cai *et al.* 1993). Since no MT are present in or near the dictyosomes in pollen tubes (Lancelle *et al.* 1987; Derksen *et al.* 1995) a function in vesicle movement (Williamson 1986) seems unlikely. Perhaps the kinesin of pollen tubes is similar to the unconventional kinesin that was localized in *Saccharomyces* at the site of polar growth (Lillie & Brown 1993) and is involved in polar growth and/or intra-membrane motions, rather than generating organelle movements (see also below on myosin). The finding of dynein-like polypeptides in *Nicotiana* pollen tubes (Moscatelli *et al.* 1994) suggests that it has a role as a cytoplasmic motor, but like kinesin, no function has been demonstrated. A MT bundling protein has been isolated by Cyr & Palevitz (1989). Recently, Schellenbaum *et al.* (1993) and Seagull *et al.* (1990) isolated a MAP that immunologically bears similarities to neuronal tau-like proteins. These τ -like proteins were localized on MT in protoplasts from tobacco pollen tubes (T.L.M. Rutten, unpublished data).

Actin filaments (AF) are abundant and show the same polar distribution as MT (Pierson & Cresti 1992). Cortical AF co-localize with the cortical MT, as seen after double labelling with anti-tubulin and rhodamine-phalloidin in *Nicotiana* pollen tubes (Pierson *et al.* 1986). These results are confirmed by observations using the electron microscope. MT are almost invariably accompanied by mostly single microfilaments and bridges between MT and microfilaments are regularly seen (plant cells, e.g. Kengen & Derksen 1991; pollen tubes, Franke *et al.* 1972; Lancelle *et al.* 1987; Pierson *et al.* 1986; Raudaskoski *et al.* 1987). In ultra-thin sections, SV can be seen in between the MT/AF, but whether this signifies a functional relationship or is caused by the geometry of the cortical region, that is exclusion from the MT/AF site, remains open. A mainly skeletal function for the cortical MT and AF is indicated by observations of J. Derksen and I. Lichtscheidl (unpublished data) who used UV irradiation to disrupt AF (Jackson & Heath 1993) and colchicine treatment to depolymerize MT. After this treatment the cytoplasm immediately broke down and the organelles moved towards the tip in an irregular fashion, pushing aside vacuoles and an unidentifiable 'ground plasm'. The wall at the tip remained intact. UV or colchicine alone did not cause this specific effect. This could mean that MT function as anchor sites for strained AF that are firmly connected to the tip (see also below). This would also explain the results from Joos and co-workers (1994, see above), as older highly vacuolized pollen tubes may be more dependent on

internal anchor sites to stabilize the AF. Protoplasts made from pollen tubes lose their cytoskeleton but, during regeneration, a series of cortical AF become apparent that bear similarities to the AF patterns seen in hydrating pollen. As these patterns arose in both karyoplasts and cytoplasts, it was concluded that the membranes of these protoplasts, and also pollen tubes, have an intrinsic capacity to organize cortical AF in well defined polar patterns (Rutten & Derksen 1990). In the same protoplasts, however, MT are only found in swirls or concentric arrays with cross bridges that are much longer than those in the pollen tube (Rutten & Derksen 1992). These MT patterns resemble the wave-like arrays seen in intact pollen tubes after cytochalasin treatment (Derksen & Traas 1984). It may be concluded that cortical MT eventually conform to existing AF arrays, but that in protoplasts this capacity is lost. This behaviour of MT and AF echoes that in neurites where extensions and leading edges are formed by AF and only later become stabilized by MT (Heidemann 1990).

The non-cortical AF in the tube mainly occur in bundles that reflect the pattern of cytoplasmic streaming (Pierson & Cresti 1992). Myosin-like peptides have been immunolocalized in pollen tubes (Tang *et al.* 1989a), but the intensity and extent of the staining is variable. Staining always seems more intense in the tip region, maybe indicating myosin on SV that cannot be seen individually. Antibodies to myosin head and tail regions bind to the plasma membrane of pollen tubes, but S-1 myosin seems only present on the surface of the vegetative cell and the generative nucleus (Tang *et al.* 1989a). Myosin or myosin-like proteins may also be present in locations where they have no obvious motor functions, as in microvilli (Mooseker *et al.* 1991) and at the site of polar growth of yeast (Lillie & Brown 1993). Therefore, it may be speculated that myosin is involved in tip growth but the data on pollen tubes are too incomplete for a further analysis. Organelle motion depends on actin–myosin interactions, as indicated by the immediate cessation of organelle movement after cytochalasin treatment (Emons *et al.* 1991; Pierson & Cresti 1992; Steer & Steer 1989) including organelle movement in isolated cytoplasm from pollen tubes (Kohno & Shimmen 1988; Kohno *et al.* 1991). No involvement of other motor proteins has been shown so far, but close contact between organelles (see above) may alter movement or even cause motion of myosin-depleted organelles (see also Pierson & Cresti 1992).

The movement of the generative cell within the pollen tube can be regarded as a special case of organelle transport, as it moves in register with the growing tip. The generative cell is engaged in intertwined bundles of cytoplasmic AF (Fig. 2F). It is possible that movement depends on dissociation of the AF bundles in front and a re-bundling in the rear, or the AF encaging the generative cell are connected to the cortical AF system and so advances with the growing tip. Based on MT depolymerization by cold shock (Åström *et al.* 1991) and colchicine (Joos *et al.* 1994; Van Aken *et al.* 1994) it was concluded that transport of the generative cell also involves MT in the generative cell (see also Cresti *et al.* 1990; Lancelle *et al.* 1987).

In pollen tubes grown *in vivo*, star-like formations of actin are seen in the tube (Pierson *et al.* 1986). They have been hypothesized to be a kind of focal adhesion site, similar to that in animal cells (Saunders & Lord 1992). However, besides a morphological similarity there is no data on their function. Moreover, it is by no means certain that they are located at the wall, as the images shown were made in conventional fluorescence microscopes. AF bundles near the tip may end abruptly, fanout, or change orientation in a way similar to the reverse fountain-like streaming pattern (Fig. 2C). Numerous AF may be seen in the tip (Åström *et al.* 1991; Pierson *et al.* 1986; Tang *et*

al. 1989b), but due to the geometry of the tip, their orientations are difficult to establish using actin-specific probes and light microscopy. Mostly a dense network (Fig. 2C) is assumed to be present (Steer 1990; Pierson & Cresti 1992). Similar networks are also visible in the electron microscope (Derksen *et al.* 1995; Lancelle *et al.* 1987; Steer & Steer 1989; Tiwari & Polito 1988), where they are seen between the SV, but also in between curling masses of faintly stained TER near to the SV, as in *Nicotiana* (Derksen *et al.* 1995). The dynamic activities in the tip region are likely to involve dynamic changes in the AF network. Disruption of the calcium gradient (see calcium section below) leads to growth inhibition and a rearrangement of the AF network in the tip (Fig. 2D,E).

Proteins that share epitopes with spectrins and have a molecular weight similar to spectrin are immunohistochemically detected at the membrane of plant cells (De Ruijter & Emons 1993), but especially in the pollen tube tip (Kengen 1992). Spectrins, or fodrins, are membrane and actin-associated proteins that restrict protein mobility in the plasma membrane and stabilize the structure, as has been shown for red blood cells (Bennett & Gilligan 1993). The presence of these cytoskeletal components at the tip suggests that they may be involved in resisting turgor pressure by reinforcing the membrane at the tip and connecting it to the AF/MT anchor sites in the tube (Steer & Steer 1989; Steer 1990). Spectrins in animal and yeast cells have been shown to contain the Src homology 3 (SH3) and Pleckstrin motifs (Bretscher 1993; Macias *et al.* 1994; Musacchio *et al.* 1993). These motifs are known to be involved in signal transduction pathways (Mayer & Baltimore 1993).

Profilin is a low molecular weight protein that sequesters actin, forming complexes so preventing actin polymerization. Profilin is involved in several other key areas of metabolic regulation, for example interaction with phosphatidylinositol 4,5 biphosphate (PIP₂), Ca²⁺-regulated pathways and poly-proline binding (review: Bretscher 1993). Profilins are present in plant cells and have been proposed to be involved in regulating AF turn over (Staiger *et al.* 1994; Valenta *et al.* 1993) and have been implicated in plant signal transduction (Drøbak *et al.* 1994). They are present in pollen and pollen tubes (Mitterman *et al.* 1994) and are believed to play an important role in AF dynamics in the tip, but their presence in the tip could not be confirmed in immunocytochemical studies (Derksen & Pierson, unpublished data). This might be due to technical problems as profilins have a low molecular weight and may be lost or redistributed during processing (e.g. Melan & Sluder 1992).

The current data show that many actin and MT-associated proteins are present in pollen tubes, supporting the contention that these cells (including the tips) have the potential for undertaking the type of cytoskeletal activities found in many animal cells, as well as those typical of other plant cells.

EXOCYTOSIS AND ENDOCYTOSIS

Aspects of endocytosis and exocytosis in plant cells have been reviewed by Morr e (1990), Low & Chandra (1994), Battey & Blackbourn (1993) and Hawes *et al.* (1991).

Exocytosis

The very first electron microscopic observations on pollen tubes showed SV accumulated at the tip (Rosen *et al.* 1965). They are transported from Golgi stacks in the tube to the tip via actin filaments. Cytochalasin treatments cause SV to accumulate in the cytoplasm (Picton & Steer 1981; Steer & Steer 1989). Video-enhanced microscopy

(Herth 1989; Pierson *et al.* 1990) shows SV as a compact mass of particles moving irregularly at the tip, as if exhibiting Brownian motion. However, actin is present in the tip and SV may carry myosin (see cytoskeleton), so directed movements may occur. SV can leave the tip and return to the streaming cytoplasm (see also Fig. 1). SV in the tip can be found alongside the plasma membrane, which probably represents a stage in docking and discharge, but in most cases they detach and again start their irregular movement (Derksen & Lichtscheidl, unpublished data). Fusion is rarely seen and seems to occur extremely rapidly (see also Fig. 1B).

Docking and fusion of SV are separate processes which, in animal cells, involve specific sets of proteins for recognition, docking and ultimately fusion (for review see Battey & Blackbourn 1993). Recently, the presence of proteins belonging to the annexin family has been established in plants. In animal cells annexins promote vesicle fusion and bind to the cytoskeleton in a calcium-dependent manner, so regulating exocytosis (Battey & Blackbourn 1993). Annexins isolated from plant cells, however, do not share the Ca^{2+} binding site and actin-binding capacity of animal annexins (Blackbourn *et al.* 1992), however, they still act in a Ca^{2+} -dependent manner (Blackbourn *et al.* 1991; Blackbourn & Battey 1993). Battey & Blackbourn (1993) have suggested that they are important for regulation of Ca^{2+} ion channel activity: docking and fusion require relatively high Ca^{2+} concentrations (Picton & Steer 1983; Steer 1988a,b, 1989; Steer & Steer 1989; Battey & Blackbourn 1993) and calcium imported at the tip (see Calcium) may fulfil this requirement.

Recently, Derksen *et al.* (1995) and Van Amstel *et al.* (1994) used serial sections of cryo-fixed and freeze substituted pollen tubes of *Nicotiana* to study SV formation. They showed that SV originated from the Trans-Golgi-Network (TGN; Griffith & Simmonds 1986, see also Staehelin 1990) that apparently splits up into vesicles that further mature into SV. Dictyosomes in the main cytoplasmic zone contribute SV to the stream of cytoplasm moving to the tip (see: Steer & Steer 1989). Dictyosomes are found associated with actin (Derksen *et al.* 1995) and, like SV, may move along with the cytoplasmic stream.

In *Nicotiana* only a single type of SV was found in serially sectioned CF-FS pollen tubes. These results were further confirmed by different types of staining on chemically fixed pollen tubes (Derksen *et al.* 1974; Van Amstel 1994). Several earlier reports claim the existence of different types of SV (Rosen *et al.* 1965; Larson 1965; Cresti *et al.* 1977; Ciampolini *et al.* 1982; Lin *et al.* 1977). These do occur in fungi, where two different types of vesicles occur, for matrix material and enzymes (Wessels 1988, 1993; Hardham *et al.* 1994), whereas in pollen tubes different secretory products must occur in a single type of vesicle.

It should be remembered that the plasma membrane at the tip is derived from the SV membranes. Thus, SV membranes could carry adherence sites for the cytoskeleton, recognition, docking and fusion proteins, CMF and callose synthases, enzymes to modify the wall after its secretion and channels to import Ca^{2+} and other ions. Except for annexins and CMF, none of these proteins have been detected on SV membranes, so they might be directly inserted into the plasma membrane.

Most observers agree that there is a high level of excess membrane secreted in the course of pollen tube growth (Picton & Steer 1981). The fate of this excess membrane may vary, between species and between growth conditions. Membranous inclusions can be found in the secondary wall of tobacco pollen tubes (Kroh & Knuiman 1985a,b), and such tubes when grown *in vivo*, contain folded plasma membranes or plasma tubules

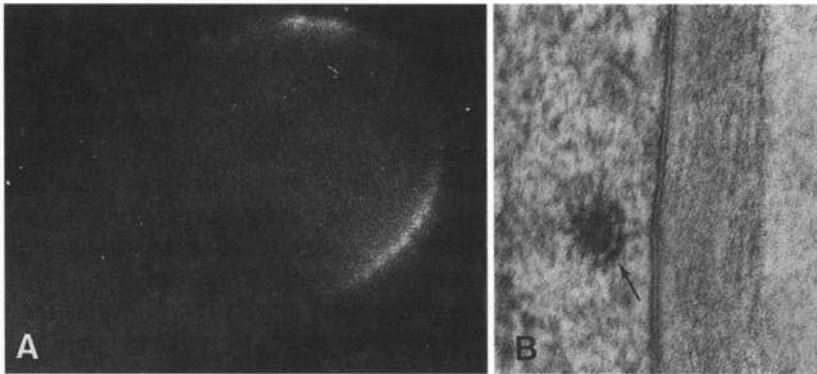


Fig. 3. (A) Immunofluorescent localization of a clathrin-like protein in the tip (see Derksen *et al.* 1985). The primary antibody (Sigma C 8034) was raised against bovine brain coated vesicles. FITC-rabbit anti-goat was used as a second antibody. The typical spotted staining at the edge of the cell indicates the presence of clathrin coated pits. Magnification: $\times 5000$. (B) Coated vesicle separated from the plasma membrane. Parts of the coat are still visible (arrow). Magnification: $\times 450\,000$.

(Kandasamy *et al.* 1988). The latter observations were carried out after conventional aldehyde fixation and were only found in tobacco.

Endocytosis

Endocytosis, the uptake of vesicles formed by the plasma membrane, may occur in several ways. Soon after their description in animal cells, coated vesicles (CV) were also seen in plant cells (Newcomb 1981) (Fig. 3). In animal cells they are regarded as carriers of specific ligands and intimately connected with signal transduction. There is a strong similarity between CV in animals and plants, though the latter never exceed a diameter of 100 nm. CV derive from coated pits (CP) that apparently spontaneously form on the membrane after triggering by a signal, for example by a hormone. As a result of the clathrin coating the CP will close, pinch off and become transported as a CV to another compartment. The typical reticulate coat is shed after detachment from the membrane. As in animals, the reticulate coat in plants is formed by the high molecular weight clathrin chains (Robinson & Depta 1988; Hawes *et al.* 1991). In addition, low molecular weight chains occur that bind Ca^{2+} -calmodulin and may become phosphorylated (Lin *et al.* 1992). Shedding of the coat is an active process (Kirsch & Beevers 1993). CV contain ATPases as an integral component (Drucker *et al.* 1993).

CV occur at different sites in plants cells (Coleman *et al.* 1988; Hawes *et al.* 1991). At the plasma membrane they occur abundantly as has been convincingly shown by means of dry-cleaving (Traas 1984). In pollen tubes smaller vesicles with a more smooth coat occur associated with the cisternae of dictyosomes (e.g. Derksen *et al.* 1995). Therefore, the coat of these vesicles may be different. They are probably involved in intra-Golgi transport (Orci *et al.* 1986), but also have been proposed to represent a second route to or from the dictyosomes (Lodish 1988; Mellman & Simons 1992). CV are involved in the directional transport of proteins from the dictyosome to other cell compartments (e.g. Hoh *et al.* 1991; Low & Chandra 1994).

Here only the endocytotic CV at the plasma membrane will be discussed. The routes and destinations of these CV are still uncertain. By analogy to animal cells they are supposed to be transported to a lysosomal compartment, i.e. the vacuole, possibly involving a partially coated reticulum and multi-vesicular bodies (MVB), or they might

be directly or indirectly transported to the dictyosome and their contents may be recycled (Low & Chandra 1994). The partially coated reticulum is thought to be functionally similar to the trans-Golgi network (Hilmer *et al.* 1988), which then would be involved in both pathways. Solid markers are taken up by CP/CV and appear in the dictyosome and the vacuole (Low & Chandra 1994). Solutes also have been reported to be endocytosed via coated pits, but the validity of these experiments is disputed (Kranz & Lorz 1990; Villanueva *et al.* 1993; Low & Chandra 1994). Receptor uptake via CP/CV is uncertain as hardly any well defined receptors are known from plant cells. However, information on elicitors and polygalacturonic acids (Horn *et al.* 1988; Low *et al.* 1993; Van Cutsem & Messiaen 1994) may be taken as evidence for receptor-mediated endocytosis.

In pollen tubes CP/CV are present at the plasma membrane (Pierson *et al.* 1986; Steer 1988a; Derksen *et al.* 1995). As in other plant cells (Kengen & Derksen 1991, 1993 and refs therein) they may be seen connected to AF/MT (Pierson *et al.* 1986; Derksen *et al.* 1995). Thus, endocytosis possibly involves both AF and MT; the latter may be an anchor site for AF (see also above). Recently, it was shown that a strong accumulation of CP occurs in a zone 6–15 μm behind the tip of tobacco pollen tubes grown *in vitro* (Derksen *et al.* 1995) and that fluorescent markers enter the tubes near the tip (O'Driscoll *et al.* 1993). These markers can be followed in discrete entities (vesicles?) as they are transported from the tip, through the cytoplasmic zone to the vacuole system of the tube. Clearly endocytosis and related cell–cell interactions in the style *in vivo* will occur for the most part near to the tip. CV have been presumed to retrieve excess membrane material delivered by exocytosis (Steer 1988a, Steer & Steer 1989). Measurements on exo- and endocytotic capacity indicate that at least a large part of the excess is retrieved (Steer 1988a; Derksen *et al.* 1995). Whether all excess membrane can be retrieved in this way is yet to be decided, as the calculations depend on assumptions that cannot be easily verified. However, the almost exclusive location of endocytosis near the tip indicates a specific function in this region. To maintain tip growth the proteins involved in recognition on docking and fusion of the SV (see above), but also the Ca^{2+} channels (see below), must be confined to the tip, or they must be inactivated or removed behind the tip (Derksen *et al.* 1995).

Confinement to the tip may involve actin and actin-associated proteins (see above). However, as the entire membrane eventually derives from SV, differences must be ultimately maintained by selective endocytosis. Therefore, specific retrieval of some membrane components like recognition proteins, annexins and calcium and other ion channels (see exocytosis, see below), but not CMF and callose synthesizing complexes (see cell wall) is inevitable. The ATPases found in CV (see above) may represent such retrieved ion channels as they seem to be inactive (Depta *et al.* 1990; Drucker *et al.* 1993). Apart from retrieving membrane components, external molecules (from the style *in vivo*) may also be received via the CP/CV at the tip. These would only be able to cross this thin cell wall near the tip, which coincides with the zone of maximum coated pit formation.

CALCIUM

In intra- and intercellular processes, Ca^{2+} is known as a second messenger capable of influencing almost all events in the cell. Calcium also plays a dominant role in pollen tube growth (see Pollen tube growth). The role of Ca^{2+} in pollen tubes has been

reviewed by Steer & Steer (1989), while its distribution has been reviewed by Herth & co-workers (1990), Pierson & Cresti (1992), Hepler & Wayne (1985) and Hepler *et al.* (1994). The significance of Ca^{2+} in pollen tube growth was recognized by Mollenhaur & Morré (1978) and the role of calcium in tip-growth was exposed by Picton & Steer (1982, 1983).

It was established in the seventies, that ionic currents flow through pollen tubes (Weisenseel *et al.* 1975). Ca^{2+} and many other ions show a tip to base concentration gradient in pollen tubes. Similar gradients of membrane-associated calcium detected with chlorotetracycline, and the calcium binding protein calmodulin (Herth *et al.* 1990) were measured. Accurate measurements are complicated by the toxic effect of chlorotetracyclin induced by irradiation. ^{45}Ca supplied in the culture medium accumulated inside the pollen tube tip (Jaffe *et al.* 1975). This accumulation was assumed to occur by an influx of the ion in the tip and uptake by a sink behind the tip (Jaffe *et al.* 1975; Weisenseel & Kicherer 1981). Further experimental evidence for the presence of Ca^{2+} channels and their role in tip growth derives from the effects of channel blockers and ionophores on pollen tube growth (Herth *et al.* 1990). A direct relationship between Ca^{2+} influx and pollen growth was further established by the use of a Ca^{2+} -selective vibrating probe (Kühntreiber & Jaffe 1990; Pierson *et al.* 1994). A positive correlation was found between the growth rate of pollen tubes and the magnitude of the Ca^{2+} influx (Pierson *et al.* 1993; Feijó *et al.* 1994). Blocking Ca^{2+} channels with gadolinium was seen to stop growth and to completely abolish the tip current (Feijó *et al.* 1994). The recent observation that addition of a hypertonic medium rapidly inhibits both growth and Ca^{2+} influx also indicates that these channels may be stretch-activated (Pierson *et al.* 1994). Stretch-activation could explain the localization of Ca^{2+} channels in the expanding part of the cell, that is the very tip (see Cell wall). The observation of sequential loss and subsequent restoration of the cytoplasmic organization after blocking Ca^{2+} channels (Reiss & Herth 1985) or dissipating the intracellular Ca^{2+} gradient with BAPTA buffers (Miller *et al.* 1992; Pierson *et al.* 1994) shows that Ca^{2+} channels are continuously replaced and/or can be rapidly deactivated. Whether stretch-activated Ca^{2+} channels exist in pollen tubes, as those described in *Saprolegnia* hyphae (Garrill *et al.* 1992), remains to be proven. Obermeyer & Weisenseel (1991) reported a gradient in Ca^{2+} channels and the involvement of calmodulin-regulated Ca^{2+} pumps. K^{+} channels have been detected in pollen tubes. The activity of at least one of the K^{+} channels seems to be under the control of cytoplasmic Ca^{2+} levels (Obermeyer & Kolb 1993; Obermeyer & Bentrup 1994). The influx of K^{+} was suggested to cause water uptake and thus to maintain turgor pressure.

In vitro growth occurs at an external Ca^{2+} concentration of between 10^{-5} and $5 \cdot 10^{-2}$ M. The optimal concentration is thought to be about 10^{-4} M (Steer & Steer 1989; Herth *et al.* 1990). Initially, the intracellular concentration of Ca^{2+} in lily pollen tubes was measured to be about $9 \cdot 10^{-8}$ M at its maximum and to slowly decline to about $4 \cdot 10^{-8}$ M at 100 μm distance from the tip (Nobiling & Reiss 1987). Later, with the use of more advanced techniques a much higher concentration and steeper gradient were recorded (Obermeyer & Weisenseel 1991; Rathore *et al.* 1991). According to the most recent measurements performed with the ratiometric dye fura-2 dextran and an advanced ion imaging system, the maximum level appears to exceed $3 \cdot 10^{-6}$ M in the very tip, whereas the basal level of less than $2 \cdot 10^{-7}$ M is reached within 30 μm distance from the tip (Pierson *et al.* 1994). These observations led to the introduction of the term 'tip-focused gradient' (Hepler *et al.* 1994) rather than tip to base gradient (Herth *et al.*

1990). Though technically difficult, the method of choice for determining cytosolic Ca^{2+} seems to be ratiometric imaging of dextran-coupled dyes (Callaham & Hepler 1991; Hepler *et al.* 1994). Because of the problem of dye saturation encountered with most dyes, including fura-2 dextran, above $3\text{--}4 \cdot 10^{-6}$ M, the question can be raised about whether these most recent measurements of Ca^{2+} concentration are still not underestimates.

The optimal external Ca^{2+} concentrations for plasma streaming in pollen tubes is about 10^{-5} M. Inhibition of cytoplasmic streaming occurs at internal concentrations of 10^{-5} M (Kohno & Shimmen 1988), which is much higher than that measured in the tip. The observed effects of electrical fields on the direction of pollen tube growth (Zejlemaker 1956; Nozue & Wada 1993; Malhó *et al.* 1994) and iontophoretic effects (Malho *et al.* 1994) show that affecting the Ca^{2+} distribution in the tip also alters growth direction (see also Pollen tube growth). It should be noted that all experiments and measurements on Ca^{2+} were carried out *in vitro* on a limited number of species. The concentration and availability of calcium in stigmatic and stylar fluids during *in vivo* growth are not known. Such information would be of great value in the context of the role of external Ca^{2+} in tube growth.

The rapid decrease in Ca^{2+} concentration behind the tip is caused by the capture of Ca^{2+} by the sink, i.e. the ER and involves a Ca^{2+} pump (Herth *et al.* 1990). Whether mitochondria additionally may act as a sink (Steer & Steer 1989 vs. Herth *et al.* 1990) cannot be decided yet (see also: Cytoplasmic organization). The massive amount of TER, and not RER, seen in the flanks of the apex in tobacco (Derksen *et al.* 1995) or even between the SV in lily (Lancelle & Hepler 1992) represent a vast sink that must be capable of dramatically decreasing the Ca^{2+} concentration behind the site of import, that is the very tip.

As calcium and its related active compounds like calmodulin are active in an astonishing range of signalling chains it is not only likely that there will be major effects of any disturbance of calcium metabolism, but also that it will be virtually impossible to directly link changes to a specific effect. However, the exceptionally high concentration of calcium at the tip shows its importance at that site for exocytosis and growth. Growth is determined by exocytosis at the tip, which in turn depends on import of calcium at that site. It is tempting to accept a direct relation between calcium import and annexin activity (Battay & Blackbourn 1993). Maybe vesicle fusion only occurs with a simultaneous import of calcium ions.

CONCLUSIONS

From the descriptions above a general picture emerges that differs at some points from earlier descriptions.

- Pollen tubes may essentially grow autonomously.
- Pollen tube growth may be directional and affected by external stimuli.
- The cytoplasmic organization in terms of zonations are relevant only for those parts directly involved in endo- and exocytosis or Ca^{2+} uptake.
- The cell wall does not regulate growth, but shows a gradient in physical properties at the tip that gives rise to the characteristic shape of the tip.
- Callose lining of the tube wall prohibits cell–cell communication, communication is restricted to the tip region.

- Turgor pressure is counteracted mainly by the cortical cytoskeleton. Possibly microtubules function as anchor sites for actin in the growing tip.
- Organelle motility and vesicle transport depend on acto-myosin interactions; reverse fountain-like streaming is a juvenile trait.
- Exocytosis determines cell growth and is in turn determined by the influx of Ca^{2+} in the tip.
- The accumulation of Ca^{2+} in the tip is transient and maintained by import at the tip and sequestering by the organelles bordering the tip.
- Import of Ca^{2+} at the tip probably occurs by Ca^{2+} channels whose activity or distribution is restricted to the site of exocytosis. Ca^{2+} channel activity may be restricted by stretch -sensitivity.
- Chemical signals emanating from the tube reach the environment via the tip only. Pectins and proteins may be the major factors.
- Signals affecting pollen tube growth must either directly effect the plasma membrane (e.g. Ca^{2+} import channels and their distribution), or they must enter via the CP behind the tip. Entrance via the fluid phase of the wall in the tip is excluded by the almost explosive growth rate at that site.

REGULATION OF GROWTH

This review has examined the structural organization of pollen tubes and the possible contribution of each component to tube growth. The previous section emphasized the diverse roles of calcium ions in this process. In this concluding section we will assess the possibilities for regulation of pollen tube growth *in vivo* by factors that are present on the stigma or in the style and ovary tissue. These female, sporophytic factors will presumably interact with one, or all three of the main systems discussed previously, i.e. wall formation and structure, cytoskeleton organization and functions, and calcium-regulated systems. We do not exclude the possibility of external factors affecting pollen tube gene expression and translation, but the evidence to date suggests that regulation of tube growth *in vitro* does not occur directly at this level. This is despite the fact that female sporophytes are highly specific in their accommodation of tube growth. Compatibility with the growing tube has to be matched, usually at the species or genus levels. In some instances this is at the gene locus level as in self-incompatibility systems, but even here the expression of genes is usually completed by the time pollination occurs.

Wall formation at the tip depends on a supply of SV and on the incorporation of their contents into the forming wall as the tip advances. Inhibition of vesicle transport to the tip can be achieved artificially, and so this may be a control point that can be regulated by external factors. However, tubes growing *in vitro* apparently lack any control over this process, the rate of secretory vesicle formation and transport to the tip remaining constant despite fourfold changes in tip extension rates induced by external factors. Extracellular modification of secreted polysaccharides (demethylation of pectins, for example) may be required to enable the components to assemble into a structurally competent wall. Interference with the requisite extracellular enzymes would be a further control point available to the female sporophyte.

Cytoskeleton organization and function are intimately related to calcium levels in the tip, so these ionic effects seem likely to dominate any regulation exerted on the

cytoskeleton system. Control of apoplastic calcium levels is a feature of all sporophytic plant tissues, so it should be expected that these control systems would be adjusted to accommodate and regulate pollen tube growth. Future work will have to be directed at examining these relationships and determining the means and magnitude of the calcium regulated growth controls. Further involvement of cytoskeletal and -associated proteins in signal transduction and growth control is expected to be demonstrated in the near future.

ACKNOWLEDGEMENTS

We thank Drs E. Pierson, R. Hochstenbach and A.M.C. Emons for critical comments. We also thank Mr B. Knuiman, Th. Laeyendecker and J. Eygenstein for technical support. F.D. was a recipient of the EC short-term training and mobility grant (GT920152) under the CAMAR programme. We also thank our colleagues for various support.

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