

# Molecular Mechanisms of Pollen Tube Growth and Differentiation

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## INTRODUCTION

The extremely reduced, three-celled, haploid male plant (male gametophyte) of flowering plants has a number of specialized functions to perform. The primary functions are the production of two sperm cells and their transport within the pollen tube through the tissues of the style and ovary into the embryo sac in the ovule, where they participate in double fertilization. The early part of the life of the male gametophyte, following meiosis, occurs within the sporophytic tissues of the anther. During this period, a number of critical developmental events occur that are preceded or accompanied by the induction of activity of a large number of genes (for reviews, see Mascarenhas, 1990, 1992; McCormick, 1991; Scott et al., 1991; Bedinger, 1992). The molecular events that take place during pollen maturation from meiosis until the time the pollen is released make up the topic of an accompanying paper (McCormick, 1993, this issue) and will not be discussed here.

At the time of anther dehiscence, the pollen grains of most flowering plants are bicellular: they contain a vegetative cell and a generative cell. Among the 265 families studied by Brewbaker (1967), 179 produced only bicellular pollen and 54 produced only tricellular pollen, which contains the products of generative cell division, the two sperm cells. Thirty-two families included both bi- and tricellular pollen-producing species. Seventy percent of the ~2000 species studied released pollen in the bicellular stage, and 30% shed their pollen as tricellular grains following sperm production. Only 10 genera were found to include both bicellular and tricellular species. In addition, all taxa considered to be phylogenetically primitive were bicellular (Brewbaker, 1967).

When the pollen grain is released from the anther, it exists as a free organism until it is carried by wind, insects, or other agents to the stigma of the pistil in a flower, where it then begins another phase of its life. If the pistil is a compatible one, the pollen grain germinates and extrudes a tube, the pollen tube, within which the sperm cells are transported to the embryo sac. During this phase of development, there is an intimate interaction of the pollen tube, a part of the vegetative cell of the male gametophyte, with the cells and tissues of the pistil. Many of the biochemical events that occur in the vegetative cell of the pollen grain prior to anthesis are related to the preparedness of the gametophyte for the demands of germination and growth of the tube in the pistil.

In most plants, pollen germination occurs very rapidly, and the rate of tube growth is extremely high. In maize, for example, pollen germination occurs within 5 min of deposition on the silk, and the pollen tube grows through as much as 50 cm of style within ~24 to 36 hr at rates close to 1 cm/hr (Barnabas and Fridvalszky, 1984).

In this review, I will discuss several topics that I consider important for an understanding of the major functions of the male gametophyte in the production of the pollen tube and the conduction of the sperm cells within the tube to the embryo sac. These topics include the regulation of gene activity; pollen tube wall structure and growth; the cytoskeleton; and the interaction of the pollen tube with the female structures in the flower, excluding incompatibility phenomena, which are discussed by Nasrallah and Nasrallah (1993, this issue) and Newbigin et al. (1993, this issue). Limitations of space do not permit me to include all the relevant information from the literature or other important topics, such as gamete competition. I have also not always attempted to reference the first study describing a particular finding:

## REGULATION OF GENE ACTIVITY IN THE GERMINATING POLLEN GRAIN AND TUBE

Pollen grains of many species germinate and grow in vitro. Protein synthesis is initiated very rapidly during pollen germination. Within 2 min of transfer of ungerminated *Tradescantia* pollen to a growth medium, there is a large increase in polysomes and a decrease in single ribosomes (Mascarenhas and Bell, 1969). Even when pollen is placed in distilled water or in a medium containing actinomycin D, an inhibitor of transcription, the shift of ribosomes into polysomes takes place. Such a rapid initiation of protein synthesis has also been seen with pollen from several other plants (Linskens et al., 1970; Tupy, 1977; Hoekstra and Bruinsma, 1978, 1979).

Various plants show wide differences in the ability of their pollen to germinate and grow in the presence of protein synthesis inhibitors. The protein synthesis inhibitor cycloheximide, although it does not prevent germination, inhibits tube growth in *Tradescantia paludosa* within the first 10 to 20 min after it

is added to the growth medium, and the total growth obtained is a fraction of that in the control (Mascarenhas, 1971, 1978b). In the pollen of *T. virginiana*, reduced dictyosome activity, as measured by vesicle production, is seen within 10 min of cycloheximide addition, although tube extension is not affected until later. After 30 min, the production of vesicles ceases completely (Picton and Steer, 1983). The insensitivity of germination and very early pollen tube growth to cycloheximide has been found in many other plant species (Shivanna et al., 1974a; Sondheimer and Linskens, 1974; Johri and Shivanna, 1977; Mascarenhas, 1978b; Nakamura, 1978; Hoekstra and Bruinsma, 1979). In most of the plants studied, which include both bi- and tricellular pollen species, the proteins required for germination are already present in the mature pollen grain, and new protein synthesis is required only for growth of the pollen tubes following germination. In plants such as *Impatiens balsamina*, blocking protein synthesis has no effect on germination or pollen tube growth (Shivanna et al., 1974b), indicating that the mature pollen grain already contains all the proteins that are necessary for germination and pollen tube growth. However, in other plants, such as *Lilium*, *Clivia* (Franke et al., 1972), and *Typha* (Hoekstra and Bruinsma, 1979), new protein synthesis is required for germination because blocking protein synthesis prevents germination from occurring.

Early work using inhibitors of RNA synthesis such as actinomycin D (reviewed in Mascarenhas, 1975) suggested that in several plants studied, germination and early pollen tube growth are not dependent on new RNA synthesis and that the RNAs required for germination and early tube growth are already present in the pollen grain at the time it is released from the anther. Biochemical analysis showed, moreover, that the mRNAs, ribosomes, and tRNAs required for germination are synthesized during pollen maturation and persist in the pollen grain until they are utilized for translation during the germination process (reviewed in Mascarenhas, 1988). In most species, the block in tube growth induced by actinomycin D occurs later than that induced with protein synthesis inhibitors (reviewed in Mascarenhas, 1975).

In summary, these and other observations indicate that the dependence of germination and early pollen tube growth on protein synthesis on presynthesized mRNA varies with the plant species. Some or all of the proteins whose activities are required for germination are already present in the mature pollen grain. In most plants that shed bicellular pollen, later pollen tube growth and the division of the generative cell into two sperm cells are dependent on new mRNAs synthesized after the pollen tube is formed (reviewed in Mascarenhas, 1975).

Although the pollen of most plants studied contains ribosomes, mRNAs, and tRNAs, new RNA synthesis does occur during pollen germination and tube growth. The evidence seems to indicate that the RNA that is synthesized is not rRNA or tRNA (reviewed in Mascarenhas, 1988). Rather, the RNAs that are synthesized during *Tradescantia* pollen germination and tube growth appear to be mRNAs or mRNA precursors (Mascarenhas et al., 1974). Based on studies of protein synthesis in the presence of actinomycin D, it was estimated that during the first hour of pollen tube growth, ~50% of the

protein synthesis utilizes previously existing mRNAs and the remaining 50% utilizes newly synthesized mRNAs (Mascarenhas and Mermelstein, 1981).

It is of interest to know whether a different set of genes or a few new genes are turned on during pollen germination or whether the same genes that were active during the terminal stages of pollen maturation continue to be expressed during germination. An analysis of protein synthesis by radioactive labeling and both one- and two-dimensional polyacrylamide gel electrophoresis and autoradiography showed no differences in bands or spots whether or not new mRNA synthesis was blocked with actinomycin D (Mascarenhas et al., 1974; Mascarenhas and Mermelstein, 1981). These results suggest that there are no qualitative differences between the mRNAs present in the ungerminated pollen grain and those synthesized during germination, at least for the more abundant mRNAs. Additional support for the similarity of pre- and postanthesis gene expression has recently been obtained by Weterings et al. (1992), who have found that the tobacco pollen-specific gene, NTPc303, is transcribed both during pollen maturation and during germination and pollen tube growth.

The enzyme phytase is synthesized during germination of petunia pollen on mRNA already existing in the pollen grain prior to anthesis (Jackson and Linskens, 1982). Two phytases with different pH optima have been found in germinating *Lilium longiflorum* (lily) pollen. One of the phytases is already present in mature ungerminated pollen; the other is newly synthesized during germination from preexisting mRNA (Lin et al., 1987). A 65-kD polypeptide is synthesized in tobacco pollen tubes but is not present in mature pollen. This polypeptide is, however, synthesized on mRNA already present in the mature pollen grain because its synthesis is insensitive to actinomycin D (Capkova et al., 1988).

The sensitivity of the two-dimensional gel electrophoresis methods used did not, however, allow the definitive conclusion that the genetic program that exists during the latter part of pollen maturation is identical to that used during germination and tube growth. It is possible that differences in these genetic programs exist, especially in genes that represent rare or moderately rare mRNAs. The successful isolation of genes expressed specifically in the pollen tube would provide conclusive evidence for a unique genetic program at this stage of male gametophyte development. Unfortunately, very little new work has been done in this important area of pollen development.

The pool of mRNAs that is present in the pollen grain at anthesis is the product of ~20,000 different genes, of which ~10% might be unique to the pollen grain (Willing and Mascarenhas, 1984; Willing et al., 1988). Many of these genes are activated late in pollen development, after the completion of microspore mitosis (reviewed in Mascarenhas, 1990). The mRNA for one such "late" pollen-specific gene from maize, Zm13, is first detectable after microspore mitosis, and the mRNA continues to accumulate until anthesis. Although the mRNA is first detectable after microspore mitosis, it is not translated until the day of anthesis, ~10 days later, and the mRNA continues to be translated during pollen germination

and tube growth (A. Scheewe, A. Bokhari-Riza, D. Crone, R. Desir, J. Rueda, T. Reynolds, M. Gutensohn, D. Hamilton, Z. Wang, M. Peja, T. Leib, J. Dias, and J. Mascarenhas, unpublished data). The translation of the Zm13 mRNA is thus repressed during pollen maturation. Results with transgenic *Arabidopsis* plants containing promoter constructs fused to the  $\beta$ -glucuronidase (GUS) reporter gene indicate that the *cis* elements responsible for the translational repression are present in the 5' untranslated region of the mRNA. One might expect many of the late mRNAs to be so regulated in their translation.

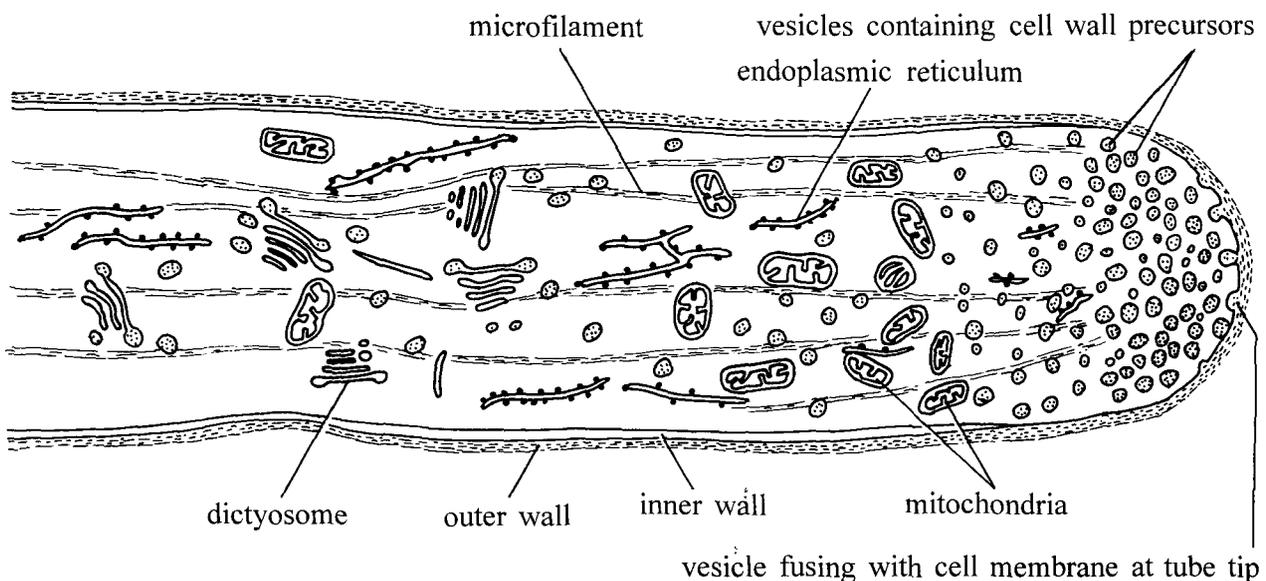
The pattern of Zm13 protein synthesis—that is, beginning on the day of anthesis and continuing during germination and growth of the pollen tube—indicates that the protein has primary functions during pollen tube growth in the style. If Zm13 is a representative example of the late class of pollen-expressed genes, then one might expect most, or at least many, of the late gene products to have primary functions in germination and growth of the tube through the style. The tentative functions, based on sequence comparisons, of several genes that have been cloned from cDNAs from mature pollen seem to support their role in pollen tube growth (reviewed in McCormick, 1991; Mascarenhas, 1992; McCormick, 1993, this issue).

#### POLLEN TUBE WALL STRUCTURE AND GROWTH, AND THE INVOLVEMENT OF THE CYTOSKELETON

The nature of pollen tube growth is different from that of most other plant cells, in which growth takes place over the entire

surface of the cell. Pollen tube growth, in contrast, is restricted to the tip region. The growth zone in lily, for example, has been estimated to be 3 to 5  $\mu\text{m}$  long (Rosen, 1961). The fine structure of the pollen tube as it relates to wall synthesis has been reviewed several times (Rosen, 1968; Heslop-Harrison, 1987; Steer and Steer, 1989; Cresti and Tiezzi, 1990; Pierson and Cresti, 1992) and hence will be summarized only briefly here. Figure 1 provides a diagrammatic picture of the important structural features near the tip region of a pollen tube.

Pollen tubes appear to contain distinct cytological zones (Cresti et al., 1977; Uwate and Lin, 1980). In *Prunus* pollen tubes, five zones have been identified: the apical growth zone, the smooth endoplasmic reticulum (ER) zone, the rough ER zone, the vacuolation zone, and the deterioration zone (Uwate and Lin, 1980). The cytoplasm of the pollen tube in the non-growing region behind the tip contains an abundance of mitochondria, dictyosomes, ER, and vesicles. The growing tip region, by contrast, contains a large number of vesicles and an absence of other organelles. The cytoplasmic structures in the pollen tube appear to be aligned parallel to the long axis of the tube except in the tip region, where they are more randomly distributed. The arrangement of membranes, mitochondria, vesicles, and other organelles seems to follow the cytoplasmic streaming patterns. There are two types of vesicle in the tip region, some 300 nm in diameter and others 50 nm in diameter (Cresti and Tiezzi, 1990). The larger vesicles are Golgi derived and contain wall precursor materials. These fuse with the cell membrane at the tip; the vesicle membranes contribute to the plasma membrane, and the contents are discharged to the outside, where they contribute to the growing tube wall.



**Figure 1.** The Growing Region of the Pollen Tube.

A diagrammatic median longitudinal section through the tip region of a growing pollen tube shows wall structure and distribution of organelles (not drawn to scale).

Confirmation for such a process of exocytosis comes from the following types of evidence. The staining characteristics of the vesicle contents and the compartmental cap that covers the growth zone at the tip are very similar (Rosen et al., 1964; Rosen and Gawlik, 1966; Van der Woude et al., 1971). Isolated secretory vesicles from pollen tubes contain polysaccharides similar to those of certain wall components (Van der Woude et al., 1971; Engels, 1974; Engels and Kreger, 1974). Moreover, immunogold labeling using an antibody specific for  $\alpha$ -L-arabinofuranosyl residues showed the presence of such residues in the outer fibrillar layer of the pollen tube wall and also in cytoplasmic vesicles (Anderson et al., 1987).

Some studies indicate that pollen tube cell walls are three layered in structure (Cresti and Tiezzi, 1990), whereas others describe a two-layered structure (Anderson et al., 1987). In the apical or growth zone of the pollen tube, the cell wall has a single layer with a lamellar appearance. In the more mature region of the tube, there is an additional inner wall layer that might be considered a secondary wall because it is formed later than the outer wall (Cresti et al., 1977; Kroh and Knuiman, 1982).

The composition and structure of the pollen tube wall are complex, and the components have not yet been satisfactorily characterized chemically. Cellulose has been tentatively identified as a component of the petunia pollen tube wall because the x-ray diffraction pattern of a residue of pollen tube walls after extraction with HCl and NaOH is similar to that of cotton hairs (Engels, 1974; Engels and Kreger, 1974). In addition to cellulose, pectic substances, hemicelluloses, and callose are also constituents of the pollen tube wall. Alkali-resistant fibrils of both crystalline  $\beta$ -1,3-glucan and cellulose associated with proteins constitute the structural polysaccharides of lily pollen tube walls (Herth et al., 1974). A purified pectic component from the tube walls of *Camellia japonica* was composed of rhamnose, arabinose, xylose, galactose, glucose, and galacturonic acid. Galacturonic acid was the major constituent, comprising 70% of the residues in the pectic component (Nakamura and Yoshida, 1980). Newly synthesized pollen tube walls of *Tradescantia* labeled with  $^{14}\text{C}$ -sucrose contained labeled glucose, arabinose, galactose, and minor amounts of other sugars (Mascarenhas, 1970). Similarly, lily pollen tube walls also consist of glucose, arabinose, galactose, xylose, and uronic acids (Labarca and Loewus, 1972). Glucose is the most abundant monosaccharide component of pollen tube walls, the second most abundant sugar being either arabinose or galactose (Van der Woude et al., 1971; Li and Linskens, 1983; Rae et al., 1985).

Studies of the glycosyl linkage composition and cytochemistry of cell walls of germinating *Nicotiana glauca* pollen suggest the presence of two major polysaccharides in the cell wall (Rae et al., 1985). These polysaccharides may be a (1 $\rightarrow$ 5)- $\alpha$ -L-arabinan with branch points through C(O)2 and a (1 $\rightarrow$ 3)- $\beta$ -D-glucan (callose) with branch points through C(O)6 and C(O)2. Immunogold labeling of *N. glauca* pollen tubes with a monoclonal antibody with primary specificity for  $\alpha$ -L-arabinofuranosyl residues showed that the arabinan is confined to the outer

fibrillar layer of the two wall layers (Anderson et al., 1987). Similarly, an antibody specific for (1 $\rightarrow$ 3)- $\beta$ -glucans was used to show that callose is located specifically in the inner wall layer, which is nonfibrillar and electronlucent in appearance (Meikle et al., 1991). The pollen tube tip does not appear to contain callose (Reynolds and Dashek, 1976; Anderson et al., 1987). The inner tube wall may, in addition to callose, contain cellulose or pectinlike materials (Kroh and Knuiman, 1982). There is, however, an unusually low level of cellulose, whose properties are not quite similar to those of celluloses from normal vegetative plant cell walls, and whether cellulose actually exists in the pollen tube wall has been questioned (Steer and Steer, 1989).

Some attempts have been made to isolate enzymes involved in pollen tube wall synthesis. A membrane system capable of synthesizing  $\beta$ -glucans was isolated from lily pollen tubes (Southworth and Dickinson, 1975).  $\beta$ -Glucan synthetase activity has been demonstrated in an enriched Golgi vesicle fraction from petunia pollen tubes (Helsper et al., 1977). UDP-glucose and not GDP-glucose was the glucose donor for this enzyme. Glucolipids do not seem to be intermediates in the synthesis of  $\beta$ -glucans in this system (Helsper, 1979). Changes in staining patterns of vesicles have been observed after the secretory vesicles have been formed by dictyosomes, suggesting that these vesicles may have a role in polysaccharide synthesis in addition to transport (Van der Woude et al., 1971).

Proteins have also been identified in the tube wall. There is indirect evidence that callose is complexed with protein (Reynolds and Dashek, 1976), and hydroxyproline-containing glycopeptides have also been found in the pollen tube wall (Cresti et al., 1977; Reynolds and Dashek, 1977; Li et al., 1983). The hydroxyproline may be associated with arabinose-containing polysaccharides (Dashek and Harwood, 1976). Estimates of the protein content of pollen tube walls are of the order of 1.5 to 3% by weight (Li et al., 1983; Rae et al., 1985). Electrophoretic analysis of the tube wall proteins of lily show several different polypeptides, many of which are different from those in the ungerminated pollen grain wall. Several of these polypeptides are glycoproteins (Li et al., 1983). Exines purified from mature maize pollen contain seven major proteins, ranging in size from 15 to 85 kD (Chay et al., 1992). One of these proteins (25 kD) is rich in hydroxyproline and also in serine and glycine, implying that it belongs to the arabinogalactan class of hydroxyproline-rich proteins. Whether identical proteins are present in the maize pollen tube wall is not yet known.

The major activity of the vegetative cell of the male gametophyte is the synthesis and assembly of the pollen tube wall. As described earlier, there are similarities and some differences in the sugar composition of the tube walls from pollen of different plants. It is indeed surprising, however, that we know so little about the biochemical processes involved in pollen tube wall synthesis. Relative to what is known about plant cell walls in general (McNeil et al., 1984; Hayashi, 1989), we have a very poor understanding of the detailed chemical structure of the different components of the pollen tube wall, their branching, cross-linking, and degree of polymerization. What are the precursors of the mature wall? How many and what sorts of

enzymes are involved in wall assembly? How are the precursors assembled? What are the functions of the proteins present in the wall? Are they structural or enzymatic? These are just a few of the many questions that require answers. We have seen earlier that as many as 20,000 different mRNA sequences are present in the mature pollen grain, and I would anticipate that many of these mRNA species must code for proteins involved in wall synthesis. Because the mRNAs in pollen are present in many more copies than are mRNAs in sporophytic vegetative cells (Mascarenhas, 1990), it should be easier to isolate mRNAs for specific enzymes required for wall synthesis from pollen than from vegetative tissues. The germinating pollen grain and growing pollen tube are attractive systems for studying wall synthesis that have not yet been adequately exploited.

## THE CYTOSKELETON AND POLLEN TUBE WALL SYNTHESIS

### The Microfilament System

Cytoplasmic streaming is extremely active in growing pollen tubes. Streaming in pollen tubes is sensitive to cytochalasin B, which interferes with microfilament function by interfering with actin polymerization. There is a direct correlation between the decrease in streaming rate and increasing concentration of cytochalasin B in the medium, thus implicating actin in this process (Mascarenhas and Lafountain, 1972). The first evidence that flowering plants contain actin was obtained with pollen of *Arum* (Condeelis, 1974). Colchicine, which causes microtubules to depolymerize, has very little effect on germination and growth of pollen tubes (Mascarenhas, 1966; Iwanami, 1968; Dexheimer, 1970), indicating that microtubules are not the major controlling elements in pollen tube growth. Based on these results, it was postulated that the contractile microfilament system is responsible for cytoplasmic streaming and possibly plays a role in the transport of cell wall precursor materials from the sites of their synthesis to the growing pollen tube tip (Mascarenhas and Lafountain, 1972). A similar conclusion was reached by Franke et al. (1972) from their observations of the effects of cytochalasin B and vinblastine (a microtubule depolymerizing agent) on lily pollen tubes.

The distribution of microfilaments in pollen grains and pollen tubes of several plant species has been visualized with rhodamine-conjugated phalloidin, which binds to F-actin (Perdue and Parthasarathy, 1985; Heslop-Harrison et al., 1986; Pierson et al., 1986; Kohno and Shimmen, 1987). Microfilament strands are distributed throughout the cytoplasm, although the extreme tip of the tube contains a dense system of microfilaments that lacks distinct strands (Perdue and Parthasarathy, 1985; Pierson et al., 1986). Cytochalasin B inhibited streaming, and the fluorescent actin strands were disrupted (Perdue and Parthasarathy, 1985). When observed

in the electron microscope, microfilaments occur in bundles that are predominantly oriented parallel to the pollen tube axis (Lancelle et al., 1987). More detailed descriptions of the actin cytoskeleton of pollen tubes can be found in recent reviews (Steer and Steer, 1989; Cresti and Tiezzi, 1990; Emons et al., 1991; Tiezzi, 1991; Pierson and Cresti, 1992).

In the ungerminated pollen grain of several plant species, actin appears to be present as large fusiform or spherical bodies in the vegetative cell. During germination, these bodies are gradually converted into fibrillar structures characteristic of the microfilament bundles of pollen tubes (Cresti et al., 1986; Heslop-Harrison et al., 1986; Tiwari and Polito, 1988, 1990a).

In some very elegant studies, Picton and Steer (1981) have quantitated the rate of vesicle production in *T. virginiana* pollen tubes when tube growth was inhibited by cytochalasin D to determine whether the vesicles could account for the observed growth rates. The closely packed vesicles in the tube tip were lost from this region in pollen tubes treated with 0.3  $\mu\text{g/mL}$  of cytochalasin D for 5 min. This observation was interpreted to indicate that although cytochalasin D inhibits the transport of vesicles, it does not affect the ability of the vesicles to fuse with the plasma membrane. In the tube region beginning  $\sim 10$  to  $12 \mu\text{m}$  back from the tip and extending for  $\sim 70 \mu\text{m}$ , in which the dictyosomes are concentrated, vesicle numbers increased with increasing length of exposure to cytochalasin D, i.e., at a rate of  $\sim 10$  vesicles per  $\mu\text{m}^3$  per 5 min. The buildup of vesicles indicated that the dictyosomes continued to produce vesicles even though they were not being transported away. Assuming that the rate of vesicle accumulation equaled the rate of vesicle production, a total of 5388 vesicles per min was calculated to be formed by a growing pollen tube (Picton and Steer, 1981). This rate of vesicle production could account for the observed pollen tube growth rates. Large amounts of membrane material are thus required for the transport of cell wall precursors to the tube tip. There appears to be a very rapid synthesis and turnover of ER, with the flow of membranes occurring from the ER to the Golgi apparatus and finally to the cell membrane (Kappler et al., 1986; Noguchi and Morre, 1991).

Actin by itself cannot cause movement of particles in the cytoplasm. Myosin, the other component of the actomyosin force-generating system, must also be involved. Convincing evidence for the role of myosin in the movement of organelles in pollen tubes was obtained by Kohno and Shimmen (1988). In internodal cells of characean algae such as *Nitella* and *Chara*, bundles of actin filaments are attached at the inner surface of the stationary chloroplast layer that lies below the cell membrane. When tonoplast-free cells are prepared and myosin-coated beads or foreign organelles are introduced into them, the beads or organelles can be induced to slide along the actin bundles (Shimmen and Tazawa, 1982; Sheetz and Spudich, 1983; Shimmen and Yano, 1984). In this model system, organelles isolated from pollen tubes showed active movement along the long axis of the algal cell. This movement was dependent on ATP and had other characteristics of being myosin dependent (Kohno and Shimmen, 1988). Pollen

tube organelles must, accordingly, contain myosin, and Kohno and Shimmen (1988) proposed that this myosin generates the motive force for cytoplasmic streaming in the pollen tube. Motility of the organelles was high at  $\text{Ca}^{2+}$  concentrations lower than  $0.18 \mu\text{M}$  but was inhibited at concentrations higher than  $4.5 \mu\text{M}$ . It was postulated that vesicles carried in the cytoplasm are deposited at the tube tip because localized higher free  $\text{Ca}^{2+}$  at the tip would inhibit actomyosin-controlled movement (Kohno and Shimmen, 1988).

Confirmation of the presence of myosin in pollen tubes has come from other experiments. When protein gel blots of tobacco pollen tube extracts were probed with monoclonal antibodies to the heavy chain of skeletal muscle myosin, a polypeptide of  $\sim 175 \text{ kD}$  was identified (Tang et al., 1989). In addition, immunofluorescence microscopy revealed small bright fluorescent spots throughout the pollen tube, with some concentration in the tip region. Based on their distribution, the fluorescent spots were thought to represent organelles and membrane-bound vesicles. The nuclear envelope of the vegetative nucleus and the outer surface of the generative cell also stained with the antibody (Tang et al., 1989). Myosin has also been localized on the surface of isolated organelles and on the surfaces of vegetative nuclei and generative cells in pollen by immunofluorescence using an antibody to bovine skeletal and smooth muscle myosin (Heslop-Harrison and Heslop-Harrison, 1989). Fluorescently labeled chicken muscle actin filaments were observed to move on a surface coated with a crude extract of lily pollen tubes, indicating that the extract contained a myosin-like translocator (Kohno et al., 1991). Finally, myosin from lily pollen tubes has been partially purified and a  $120\text{-kD}$  component tentatively identified as the heavy chain of myosin (Kohno et al., 1992).

It is now clear that the actomyosin system in the pollen tube is primarily accountable for cytoplasmic streaming and the resulting growth of the pollen tube. It would be very worthwhile to isolate the myosin gene expressed in pollen and to compare its amino acid sequence with myosins from other plants and other organisms in order to determine evolutionary relationships. No plant myosin gene has yet been isolated. Are different myosin genes active in pollen and vegetative tissues? Pollen tubes show very active streaming and, accordingly, might be expected to be a rich source of myosin and other contractile proteins and their mRNAs. Isolating cDNAs for these proteins from pollen might thus be easier than from other plant tissues.

Calcium ions are vital in pollen tube growth and in cytoskeletal function, and there is a large body of literature on the regulation of these processes by calcium. The role of calcium in pollen function is an extremely important topic but, unfortunately, limitations of space preclude its inclusion here. The reader is, however, referred to an extensive review by Steer and Steer (1989) and a recent publication by Miller et al. (1992) for information on calcium ions and their importance in pollen tube growth.

## The Microtubule System

In the pollen tube, microtubules are located mainly in the cortical cytoplasm below the vegetative cell plasma membrane throughout the length of the tube, in orderly, primarily axial arrays. They have not been detected, however, in the extreme tip of the tube. (See Pierson and Cresti, 1992, for a more detailed literature review and description.) Tubulin is present in the mature ungerminated pollen grain, but the tubulin molecules do not appear to be assembled into microtubules. They are visible only some time after hydration and activation of the grain during the germination process (Tiware and Polito, 1990b; Tiezzi, 1991).

Microtubule depolymerizing agents, as discussed earlier, have no major effect on pollen germination, cytoplasmic streaming, or pollen tube growth. What the role of the microtubules in vegetative cell function is a puzzle at present. It has been suggested that the microtubules form the relatively rigid support on which the network of actin filaments rests (Pierson and Cresti, 1992).

Double immunogold labeling of tubulin and actin in pollen tubes of *N. alata* showed microfilaments aligned parallel to many of the cortical microtubules (Lancelle and Hepler, 1991). Microfilaments and cortical or cytoplasmic microtubules are often cross-linked to one another (reviewed in Pierson and Cresti, 1992). The microtubules also appear to be linked to the plasma membrane by cross-bridges (Franke et al., 1972; Lancelle et al., 1987).

Some indication of the changes in cytoskeletal networks that occur in the pollen grain prior to and during germination has been obtained from a model culture system of lily pollen protoplasts (Tanaka and Wakabayashi, 1992). In freshly isolated protoplasts, there was an extensive network of very small actin filaments and disorganized microtubules in the central cytoplasm of the vegetative cell. Cortical actin filaments and microtubules gradually became organized as the cell wall was regenerated. The protoplasts changed from a spherical to an ellipsoid shape after 5 to 6 days in culture, and before the actual shape change, the cortical actin filaments became aligned so that they extended between the two opposite poles. With the change in cell shape, additional actin filaments formed transverse to the long axis of the cell. In contrast, cortical microtubules gradually became organized so that at 5 days of culture they formed a random network. Unlike the microfilaments, the microtubules did not show any ordered arrangement in ellipsoid cells after 6 days in culture. The protoplasts germinated and formed a tube after 8 to 10 days in culture. Just before germination, an ordered arrangement of cortical microtubules was first seen, and it paralleled that of the actin filaments. Cytochalasin B led to the disruption of both the cortical microfilaments and the cortical microtubules, whereas colchicine disrupted the cortical microtubules only. In this system, both cytochalasin and colchicine prevent germination. Based on these observations, Tanaka and Wakabayashi (1992)

suggested that the actin filaments play the dominant role in germination; the microtubule arrangement is also necessary for germination because it stabilizes the cell polarity established by the actin arrangement.

Although anti-microtubule agents have no major effects on pollen tube growth, Steer and Steer (1989) reported that pollen tubes treated with such agents show abnormal, irregular outlines. If microtubules stabilize the actin cytoskeleton, it would seem reasonable that in the presence of agents such as colchicine, the actin skeleton might not be as stably oriented, and this would result in laying down of an irregular wall.

A polypeptide showing properties common to kinesin, a microtubule-based motor molecule, has recently been identified in tobacco pollen tubes (Tiezzi et al., 1992). An antibody to calf brain kinesin identified two polypeptides of 108 and 100 kD in blots of pollen tube extracts. In addition, immunofluorescence microscopy showed staining of spherical particles at the tube apex. The presence of microtubules and the microtubule motor kinesin in pollen tubes indicates that microtubules have a function(s) in pollen tube growth. Discovering this function poses a major challenge.

## POLLEN TUBE-STYLE INTERACTIONS

### Extracellular Matrix in the Style

Pollen tubes interact intimately with the tissues of the pistil. The more general aspects of this interaction have been reviewed recently (Herrero, 1992). I will restrict myself to a discussion of the extracellular matrix (ECM) in the transmitting tissue and its possible involvement in pollen tube elongation. The transmitting tissue is the tissue in the style through which pollen tubes grow. Sanders and Lord (1989) have made the intriguing proposal that the growth of the pollen tube is a special case of cell migration in plants. They consider the tip of the pollen tube "analogous to a migrating cell, which leaves a trail of cell wall behind" (Sanders et al., 1991). Small latex beads of a diameter similar to that of pollen tubes, when placed on the stigma or cut stigma end of three plant species, *Hemerocallis flava*, *Raphanus raphanistrum* (radish), and *Vicia faba*, were observed to be translocated to the ovary at rates similar to the growth rates of the pollen tubes. The beads were translocated on the transmitting tissue, and the numbers of beads translocated were similar to the numbers of pollen tubes growing through the style in that plant species (Sanders and Lord, 1989).

It is difficult to interpret the significance of the movement of inert latex beads in the transmitting tissue and to relate the phenomenon to pollen tube growth. Based on knowledge of the role of the ECM and substrate adhesion molecules (SAM) in animal cell spreading and movement, however, it was proposed that the stylar ECM actively facilitates pollen tube

extension and "may actually interact with the pollen tube as it forms at the tip and may guide the tube toward the ovule" (Sanders and Lord, 1989). In support of the hypothesis that the stylar ECM plays a critical role, Sanders et al. (1991) used an antibody to human vitronectin, a SAM, and found vitronectin-like proteins localized in the ECM on the surface of the transmitting tissue in the style. They proposed that a molecule such as vitronectin in the transmitting tissue is involved in pollen tube growth, or, rather, according to their hypothesis, "cell migration."

This is a fascinating hypothesis that, if correct, provides a different perspective on the mechanisms that might regulate pollen tube growth. Will the consideration of pollen tube elongation as cell movement rather than cell growth, however, be supported by further work? An important argument for considering the growing pollen tube as a moving cell is the commonly held assumption that the volume of pollen tube cytoplasm stays constant during pollen tube extension and that older parts of the tube are blocked off by callose plugs. I am not aware of any really critical measurements that unambiguously demonstrate this. *Petunia* pollen tubes growing through compatible styles exhibit two phases of growth. The first consists of a slower growth rate and the second of a more rapid growth rate. At 20°C, the transition from the first to the second phase occurs between 7 and 11 hr after pollination, when the tubes are 2 to 3 mm long. What is of interest is that callose plugs are not formed in the tubes until the time of this transition (Mulcahy and Mulcahy, 1982). Better information concerning callose plug deposition and good measurements of the volume of cytoplasm in pollen tubes need to be obtained in order to better evaluate the hypothesis that growth of the pollen tube is analogous to cell migration. It should be relatively easy to make such measurements with the confocal microscope. Even though pollen tubes normally extend through the stylar ECM, the ECM is obviously not essential for pollen tube growth or for the movement of the vegetative cell nucleus or the generative or sperm cells within the tube, because pollen tubes of several plants grow well in vitro, including in liquid medium where there is no ECM. It will be interesting to see whether molecules such as vitronectin in the ECM in the pistil are truly involved in pollen tube elongation and, if so, exactly what their roles are.

### Chemotropism

Pollen tubes penetrate the stigma and grow through the style and ovary and accurately find and enter the micropyle of the ovule. What directs pollen tube growth in the pistil? Are there chemical signals involved, or are the anatomy and morphology of the pistil tissues such that, merely by elongating, the pollen tube tip will automatically arrive at the micropyle? Pistil structure and chemotropism both appear to play roles. The topic of chemical direction of pollen tube growth has a long

history, with Molisch (1889) providing the first evidence of its involvement. The early literature on this fascinating topic was reviewed by Mascarenhas and Machlis (1962b) and Rosen (1975). In *in vitro* assay systems, ~50% of the plants tested have shown directed growth of pollen tubes to various pistil tissues, presumably to chemicals diffusing from these tissues, so the phenomenon is fairly widespread (Mascarenhas and Machlis, 1962b). Although several different compounds have been shown to have chemotropic activity for pollen tubes in different plant species, no universal chemotropic substance has yet been identified. Some of the compounds reported as being active were calcium ions (Mascarenhas and Machlis, 1962a, 1964) and amino acid and amine mixtures (Schildknecht and Benoni, 1963a, 1963b).

Early workers postulated a continuous gradient of a chemotropic substance(s) from the stigma to the micropyle of the ovule. No one has yet demonstrated such a continuously increasing gradient, and arguments against such a gradient operating have been presented (Iwanami, 1959; Mascarenhas, 1978a; Heslop-Harrison, 1986; Booy et al., 1992). These arguments are based on the structure of the transmitting tissue through or over which the pollen tubes grow and on experiments that show that pollen grains placed in the center of hollow styles such as that of lily germinate and grow tubes in both directions, toward the stigma and toward the ovary. Such bidirectional growth would not be expected if a gradient of a chemotropic substance were present in the style. A gradient of a tropic compound would need to be present only in the relatively restricted regions of the pistil in which sharp changes in direction occur, for example, from the placenta to the ovule and from the ovule into the micropyle (Mascarenhas, 1978a).

Recent studies with pearl millet (*Pennisetum glaucum*) have provided evidence for pollen tube chemotropism to glucose, calcium, and a water-soluble, low molecular weight protein (~14 kD) from ovaries (Reger et al., 1992a). Pellets obtained after centrifugation of pearl millet stigmas in water elicited directional growth of pollen tubes *in vitro* on sucrose-containing medium. The pellets showed invertase activity, and yeast or tomato invertases could also induce the tropism. It was postulated that the invertase hydrolyzed the sucrose in the medium, producing glucose, which was responsible for the directional growth of the pollen tubes (Reger et al., 1992b). This is an interesting finding, but the task remains of demonstrating that glucose is indeed involved in directional growth of pollen tubes *in vivo*.

Unlike chemotropism, where the influence on pollen tube growth is positive, negative chemical influences might also operate in the pistil. When *N. alata* pollen grains are placed within cuts in styles, they germinate and produce pollen tubes that grow equally toward the stigma or ovary. If, however, the stigma is pollinated at the time the pollen is introduced into the cut, the growth of the pollen tubes toward the stigma is impeded significantly (Mulcahy and Mulcahy, 1987). This suggests that some chemical signal must travel from the pollinated stigma to the region in which the pollen grains are placed to inhibit the growth of the tubes pointed toward the stigma.

## FLAVONOLS AND POLLEN FUNCTION

In the not too distant past, pigments in pollen grain walls were generally not considered to play any important role in male gametophyte development or function. They were assumed to function mainly as insect attractants in pollen dispersal, although there were a few early reports of the stimulation of pollen germination and tube growth by flavonoids (Sedgley, 1975, and references therein). Recent evidence, however, indicates that some flavonols are essential for pollen function.

Fairly large quantities of flavonoids and other phenylpropanoids are present in the cavities of the exine of several plant species (Wiermann, 1968). The synthesis of phenylpropanes, chalcones, flavonols, and anthocyanins occurs at different periods during postmeiotic pollen development in tulip anthers (Wiermann, 1979). The bulk of the activity of most of the key enzymes of phenylpropanoid metabolism, such as phenylalanine ammonia-lyase, chalcone synthase (CHS), and cinnamic acid 4-hydrolase, is found in the tapetal plus anther locule fraction as compared to the pollen fraction. These enzymes thus appear to be produced by the tapetal cells and possibly secreted into the anther locule; they are active either in the locule or in the exine cavities of the developing microspores (Herd et al., 1978; Wiermann, 1979; Kehrel and Wiermann, 1985; Beerhues et al., 1989). Further evidence for the tapetal origin of these enzymes comes from the demonstration that in transgenic petunia plants carrying a CHS gene (*chiB*) promoter coupled to a GUS reporter gene, GUS accumulates in the tapetum (van Tunen et al., 1990).

The primary constituents responsible for the yellow color of maize pollen are flavonols (Coe et al., 1981). White pollen in maize is produced by plants carrying recessive alleles of two genes, *c2* and *whp*, both of which encode CHS. Genetic evidence shows that pigmentation is determined by the genotype of the sporophyte, as would be expected if the tapetum were responsible for pigment synthesis. The deposition of flavonoids in the pollen grain is essential for normal pollen function because although white pollen grains seem normal in all other aspects, they are unable to effect fertilization (Coe et al., 1981).

CHS catalyzes the first step in flavonoid biosynthesis. Both a *chs* cosuppressed petunia mutant created by transformation with a wild-type *CHS* gene (Taylor and Jorgensen, 1992) and the white pollen maize mutant that lacks CHS activity (Coe et al., 1981) are deficient in flavonoids. In addition, both produce otherwise apparently normal pollen in which germination and/or tube growth are inhibited. The pistils of CHS-deficient plants are, however, normal: they set seed when pollinated with wild-type pollen. The placement of CHS-deficient petunia pollen on wild-type stigmas also results in seed production (Mo et al., 1992; Taylor and Jorgensen, 1992). Transgenic petunia plants containing an antisense CHS gene are also male sterile (van der Meer et al., 1992). These results suggest that some type of flavonoid is essential for normal pollen function but that this flavonoid can be provided by either the pollen or the stigma.

Pollen germination can be restored to the CHS-deficient petunia mutant by an aqueous extract of wild-type pistils that has been shown to contain kaempferol (Mo et al., 1992). Micromolar quantities of kaempferol added to the germination medium or to the stigma before pollination are sufficient to restore wild-type germination and seed set to the mutant pollen. Representatives of all the major classes of flavonoids were assayed for the ability to restore normal function to mutant pollen, and flavonols were the only class of compound that was effective. Flavonols could also rescue the maize white pollen mutant. The activity was restricted to the flavonol aglycones; glucoside derivatives were inactive (Mo et al., 1992).

Interestingly, the flavonols quercetin, kaempferol, and myricetin, but not other flavonoids, when added to the medium at concentrations of 0.15 to 1.5  $\mu$ M, promoted germination and tube growth of in vitro-matured pollen and seed set after pollination (Ylstra et al., 1992). Pollen produced by the in vitro culture of immature pollen of tobacco and petunia normally neither germinate as well nor induce seed set as well as normally produced pollen.

Analyses of extracts of anthers and stigmas of wild-type petunia plants show no accumulation of flavonol aglycones at any stage in either organ (Pollak et al., 1993). However, glycosides of kaempferol and quercetin appear to be present. Pollak et al. (1993) have proposed that specific glycosidases must be present in anthers and stigmas that regulate the quantity of aglycones produced.

Flavonoids have been implicated as developmental signaling molecules in plant-bacterial and plant-plant interactions (for reviews, see Rolfe and Gresshoff, 1988; Lynn and Chang, 1990; Zambryski, 1992). It is conceivable that they may have such a role in pollen germination and tube growth, but the details of the process still remain to be identified. It will be interesting to see how this story unfolds in the next few years.

## CONCLUSIONS

During the past few years, there has been an almost explosive increase in the number of laboratories engaged in investigations of reproductive development in plants because of its relevance to plant biotechnology. Much of this interest has been focused on the male gametophyte. During this period, emphasis has been placed on constructing cDNA libraries and isolating genes uniquely expressed in developing pollen. A large number of genes are expressed in pollen and appear to have functions in pollen tube growth. A major challenge for the future is to determine exactly what these functions are. Thus far, no genes critical to the regulation of pollen development and function have been isolated. The power of genetics and the approach of using tagged mutants are just beginning to be exploited in the study of the male gametophyte (see Chaudhury, 1993, this issue). I have attempted to present problems pertaining to the male gametophyte and its interaction

with female tissues that, although fascinating, have not been adequately studied using the approaches of modern biology, with the hope that new individuals with backgrounds in genetics and molecular biology might find these problems sufficiently intriguing to wish to study them.

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