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Signaling and the Modulation of Pollen Tube Growth

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INTRODUCTION

Pollination in flowering plants begins when pollen lands on a stigma. The process continues as the pollen germinates and grows through intercellular spaces in the pistil, and it culminates when the pollen reaches the ovary and fertilization occurs. This complex and hazardous process involves many interactions, including cell–cell recognition and intracellular and intercellular signaling, as well as many other factors that remain to be identified.

Events occurring during pollination are assumed to be under tight genetic and cellular control because pollen tube growth needs to be regulated both spatially and temporally. Moreover, pollen tubes are a highly specialized cell type, comprising a generative cell, which contains the two sperm cells, and the vegetative nucleus. Thus, a pollen tube in fact contains a cell within a cell, which is itself haploid. This is a unique structure that is related to the singular biological role of pollen—to effect fertilization.

The processes that control pollen tube growth and that regulate and guide pollen tubes during their journey toward the ovary are of considerable interest. These topics are regarded as important both for fundamental studies of the control of fertility and reproduction in plants and also as an attractive model system for the investigation of polarized tip growth, cell–cell interactions, and signal transduction. Furthermore, the potential future application of molecular tools in efforts to control some of these processes generates additional interest in pollination.

Significant steps forward in our understanding of some of the components and mechanisms involved in regulating certain aspects of pollen tube growth have been made over the last 10 years. These advances have been facilitated by the application of an impressive array of experimental tools. Early on, it was established that pollen tubes can be grown in vitro quite readily. Coupled with the advent of live-cell imaging techniques, investigations into the factors that affect pollen tube growth have reached an impressive level of detail. Although many of these recent investigations have focused on establishing the properties of normally growing pollen tubes, studies on the signals involved in the reorientation and inhibition of pollen tube growth have also received much interest. Other studies have focused on the specific inhibition of pollen tube growth during the self-incompatibility (SI) response.

In this review, I concentrate on how our knowledge of pollen tube growth, in particular the signals that regulate pollen tube growth and guidance, has advanced over the past 10 years or so. The most notable advances have been made in the study of Ca^{2+}-regulated processes, especially the regulation of pollen tube growth by Ca^{2+}, and so these topics form a major part of this review. However, other signaling components are increasingly recognized as also playing important roles.

WHERE IT BEGINS: POLLEN HYDRATION AND GERMINATION

When it lands upon an appropriate stigma, pollen hydrates. Pollen hydration is not easy to study in vitro, although recent evidence suggests that hydration may be regulated by an aquaporin (Ikeda et al., 1997; see also Chrispeels et al., 1999, in this issue). Moreover, several detailed analyses by Heslop-Harrison document the alterations that occur in the actin cytoskeleton of pollen as it hydrates and germinates (see, e.g., Heslop-Harrison and Heslop-Harrison, 1992), some of which may underlie developmental changes. For example, once it is hydrated, the pollen grain attains a distinct polarity and germinates to produce a pollen tube, which grows by tip extension.

It is thought that pollen coat proteins, waxes, and lipids initiate signals required for adhesion and germination. Indeed, mutations that provoke alterations in the waxy cuticle can result in defective pollen and impaired fertility. For example, the *cer* and *pop-1* mutants, in which the mutant pollen fails to hydrate on wild-type stigmas (Preuss et al., 1993; Hülskamp et al., 1995a), lack long-chain lipids that are present in the tryphine layer of wild-type pollen grains. These data suggest that long-chain lipids act as signals to stimulate hydration, a contention supported by evidence indicating that specific lipids play a role in providing a directional supply of water that is important for pollen tube penetration of the stigma (Wolters-Arts et al., 1998).

In addition to lipids, there are also data implying that flavonols play an important role in pollen germination (Mo et

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al., 1992). Many of the pollen-specific genes that have been identified (reviewed in Taylor and Hepler, 1997) are expressed in mature pollen, and mature pollen grains of many species contain all the mRNAs that are required for their germination and for pollen tube growth (Mascarenhas, 1975). This has led to the suggestion that some pollen proteins may play an active role during germination and growth. Characterization of these "late" pollen-specific genes should help elucidate some of the processes required for pollen tube growth.

HOW IT IS MADE: POLLEN TUBE GROWTH

A diagrammatic representation of the basic structure of a "typical" pollen tube is given in Figure 1 (reviewed in Mascarenhas, 1993; Bedinger, 1994; Derksen et al., 1995; Taylor and Hepler, 1997). Briefly, the cytoplasm is located toward the growing tip of the front region of the pollen tube. It carries the vegetative and sperm cells, the latter of which contain the generative nuclei, often as a complex. Many other organelles, including mitochondria, Golgi complexes, and endoplasmic reticulum (ER), are also contained in this region, as are cytoskeletal components.

The cytoplasm is separated from the remainder of the pollen tube by a callose plug. These plugs are laid down at regular intervals as the pollen tube grows. During growth, the regions behind the callose plugs become vacuolated so that the cytoplasm remains concentrated in the front portion of the pollen tube, regardless of its length. At the extreme tip of the pollen tube is the "clear zone." The identities and activities of components present in this zone have not been fully established, and at present these remain controversial topics.

Rather more is known about the pollen tube wall, which comprises an outer fibrillar layer that is mainly composed of pectin, hemicellulose, and cellulose, and a second, inner layer of callose (see Mascarenhas, 1993). The callose lining is absent from the pollen tube tip. It is thought that pectins are polymerized and esterified within the Golgi complex and then transported and deposited at the growing wall by secretory vesicles. Pectins are then deesterified and cross-linked by Ca^{2+}, resulting in a rigid framework that provides support for the growing tube.

Vesicles (also called p-particles) carrying pectin and many other cell wall components, are transported to the tip via highly active cytoplasmic streaming and are incorporated into a zone of elongation in the apical dome of the pollen tube tip. Thus, the pollen tube extends its length by apical growth in a relatively small region, a mode of cell elongation and expansion very different from that of other plant cells. The tip-growing habit of pollen tubes appears (at least superficially) to be typical of other cells that extend by tip growth, such as fungal hyphae and root hairs, and there has been a trend recently to attempt to find a unifying theory that encompasses regulation of polar and apical growth of plant cells. However, it is becoming clear that although there are many parallels, the "cues" controlling tip growth are likely to vary from system to system.

An important characteristic of the growing pollen tube is the highly active cytoplasmic streaming. Because a functional actinomyosin-based cytoskeleton is required for cytoplasmic streaming and movement of organelles, it is thought that the cytoskeleton plays a major role in the modulation of pollen tube growth (Mascarenhas and Lafountain, 1972). The organization and possible roles of the cytoskeleton have been reviewed recently by Cai et al. (1997) and so are not considered in detail here. Actin and myosin-like motor proteins, which are present in pollen tubes (Perdue and Parthasarathy, 1985; Heslop-Harrison and Heslop-Harrison, 1989; Tang et al., 1989), possess functionally analogous activities to their homologs in vertebrate cells (Kohno et al., 1991; Yokota and Shimmen, 1994). However, their exact localization remains the subject of considerable debate (Miller et al., 1996).

More recently, purification of actin from pollen has allowed some of its physicochemical properties to be charac-

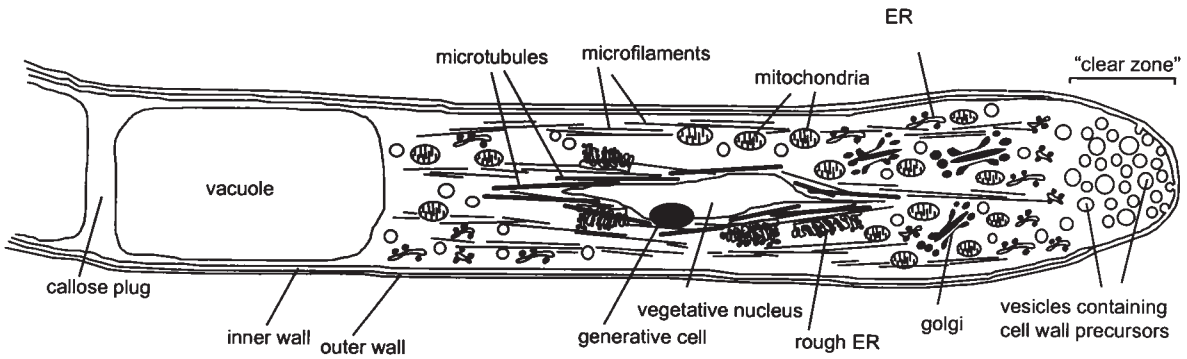

Figure 1. Generalized Diagrammatic Representation of a Pollen Tube.

Features and components generally considered to be present at the growing tip of pollen tubes. For details, see the text.

terized in vitro and in vivo (Ren et al., 1997). Furthermore, several actin binding protein families, for example, actin depolymerizing factors and profilin, have been identified and cloned in pollen tubes (Staiger et al., 1993; Mitterman et al., 1995; Lopez et al., 1996; Vidali and Hepler, 1997; Yu et al., 1998). Although it is thought that pollen profilins play an important role in pollen tube growth by regulating microfilament formation, a thorough understanding of the regulation of actin dynamics and the exact role of the cytoskeleton in pollen tube growth is currently lacking. Although it is known that pollen tubes possess microtubules and their motor proteins, depolymerization of the microtubules does not have a major effect on pollen tube growth. Because microtubules are associated with the generative cell, it has been suggested that they are involved in the regulation and organization of the generative cell/vegetative nucleus complex (see Cai et al., 1997).

There is increasing evidence that the cytoskeleton, as well as having a structural role, may also have a signaling role. In many eukaryotic cells, actin binding proteins function as stimulus–response modulators, translating signals into alterations in cell architecture. The concept that pollination involves a complex interplay between signaling pathways and components of the actin cytoskeleton has been suggested by a number of researchers, and this concept is beginning to acquire some experimental support. Recent data, albeit in vitro, suggest that profilin, which is known to bind phosphatidylinositol-(4,5)-bisphosphate (Ins[4,5]P_2) as well as actin, may play a signaling role in pollen. For example, when profilin interacts with soluble signaling components, there is a dramatic modulation of the activity of components that affect the phosphorylation of several pollen proteins (Clarke et al., 1998). This indicates a possible role for profilin in signaling pathways that could regulate pollen tube growth. No doubt in the future, the evidence for profilin's role in signaling will become stronger, and it will be possible to relate profilin-mediated signals to alterations in pollen tube architecture. Further functional analysis of recombinant cytoskeletal proteins, together with studies of fluorescent analogs, should help to unravel the roles of the cytoskeletal elements, their interactions with other pollen tube components, and their part in regulating pollen tube growth.

HOW IT GETS THERE: Ca^{2+} AND POLLEN TUBE GROWTH

That Ca^{2+} is an essential requirement of pollen tube growth has been appreciated for many years. Experiments using $^{45}Ca^{2+}$ demonstrated that Ca^{2+} is taken up by pollen (Jaffe et al., 1975; Bednarska, 1989), and inhibition of Ca^{2+} uptake results in the rapid arrest of pollen tube growth. It is also well established that Ca^{2+} plays a key role in the regulation of pollen tube growth (reviewed in Steer and Steer, 1989; Derksen et al., 1995; Feijo et al., 1995; Taylor and Hepler, 1997). Indeed, there is now convincing evidence for a positive correlation between changes in the concentration of cytosolic free calcium ($[Ca^{2+}]_i$) and changes in the rate and direction of pollen tube growth. The most important data in support of this correlation have come from the direct imaging of $[Ca^{2+}]_i$ in living pollen tubes, as illustrated in Figure 2.

Apical Ca^{2+} Gradients in Growing Pollen Tubes

Several independent studies using ratiometric Ca^{2+} imaging of pollen have established that normally growing pollen tubes possess a steep tip-focused $[Ca^{2+}]_i$ apical gradient (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Malhó et al., 1994; Pierson et al., 1994, 1996; Franklin-Tong et al., 1997; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). As illustrated in Figure 2A, a high apical concentration of $[Ca^{2+}]_i$ occurs in growing pollen tubes, but it is not detected in pollen tubes that are not growing. Moreover, treatments that eliminate the apical $[Ca^{2+}]_i$ gradients result in the reversible inhibition of pollen tube growth (Rathore et al., 1991; Pierson et al., 1994; Li et al., 1996; Malhó and Trewavas, 1996). It is now generally accepted that apical $[Ca^{2+}]_i$ gradients are likely to be a fundamental phenomenon that is common to all growing pollen tubes.

Considerable evidence from a variety of experimental approaches supports the idea that the apical $[Ca^{2+}]_i$ gradient results from localized Ca^{2+} influx through active Ca^{2+} channels at the pollen tube tip (Weisenseel and Jaffe, 1976; Kühtreiber and Jaffe, 1990; Malhó et al., 1994, 1995; Pierson et al., 1994; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). Both ratiometric imaging and measurements of extracellular Ca^{2+} influx indicate that Ca^{2+} entry is restricted to a small region associated with Ca^{2+} channel activity at the extreme apex of the pollen tube (Pierson et al., 1996). It is generally thought that when growth ceases, the apical gradient is dissipated as these channels close. The nature of these pollen tube Ca^{2+} channels is currently not known. There is speculation that they may be stretch activated (Pierson et al., 1994; Malhó et al., 1995), but further work is required to determine whether or not this is the case.

The apical $[Ca^{2+}]_i$ gradient is very steep and is accepted to be ~2 to 10 μM at the tip, dropping to basal levels of ~200 nM within ~20 μm behind the apical region (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Malhó et al., 1994; Pierson et al., 1994, 1996; Franklin-Tong et al., 1997). The dissipation of $[Ca^{2+}]_i$ behind the apex is thought to be regulated by Ca^{2+}-ATPases, which are likely to be located on the tubular ER close to the apical region of pollen tubes (Figure 1; Obermeyer and Weisenseel, 1991; Lancelle and Hepler, 1992; see Sze et al., 1999, in this issue). This organelle represents a large sink, and it is capable of rapidly sequestering Ca^{2+}. Although there are low-level fluctuations in $[Ca^{2+}]_i$ in the "shank" region of the pollen tube (Figure 1), they are generally within basal levels.

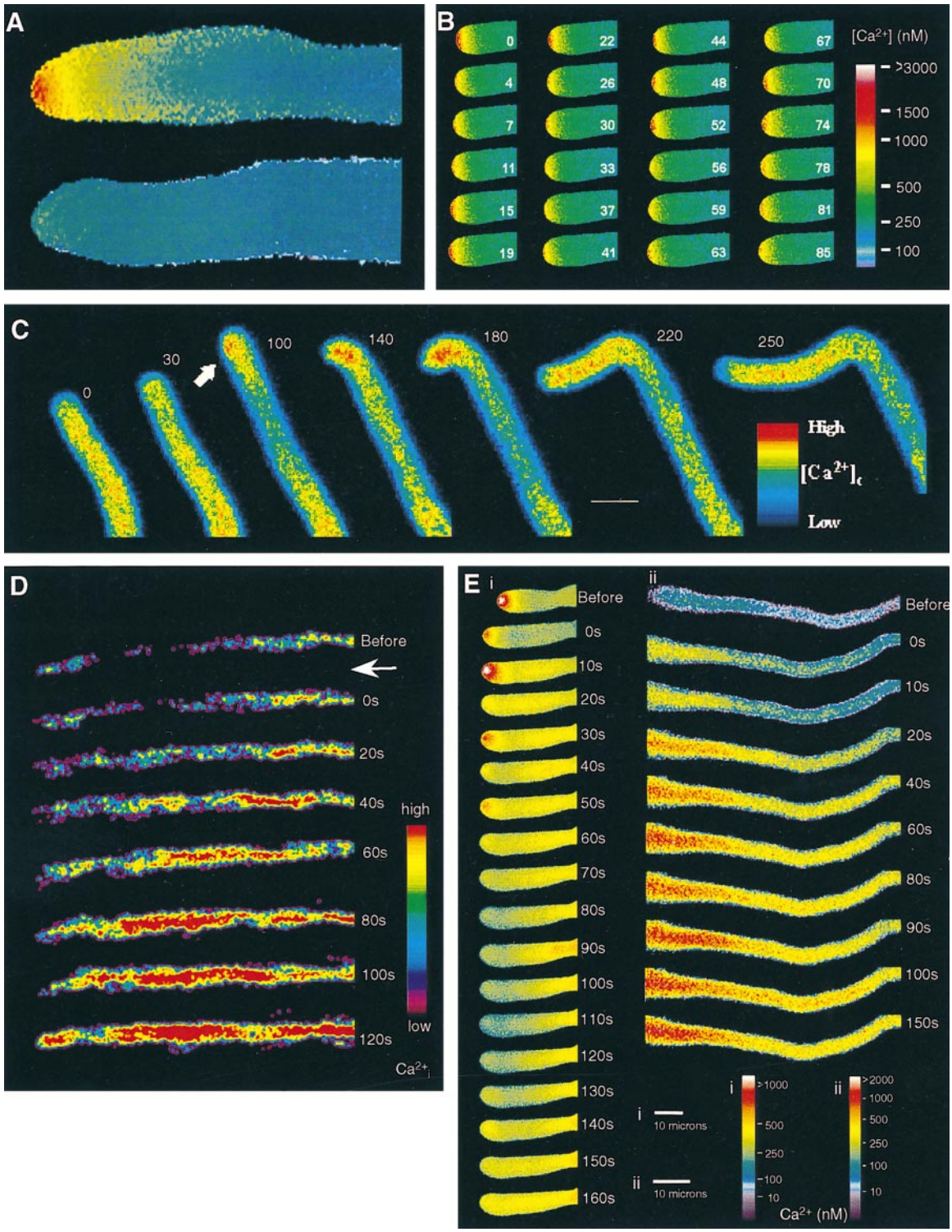

Figure 2. Imaging of Changes in Cytosolic Calcium ($[Ca^{2+}]_i$) in Pollen Tubes.

(A) Growing and nongrowing pollen tubes. Ratio imaging of $[Ca^{2+}]_i$ in a growing pollen tube of *Papaver rhoeas* microinjected with fura-2 dextran. The top image shows a growing pollen tube, with a high apical $[Ca^{2+}]_i$ gradient. The lower image shows a nongrowing tube displaying no apical

Apical Ca^{2+} Oscillates in Growing Pollen Tubes

Recent observations, made by using both ratiometric imaging and photon counting of the photoprotein aequorin, have provided good evidence that apical Ca^{2+} in growing pollen tubes oscillates over time, as illustrated in Figure 2B (Pierson et al., 1996; Calder et al., 1997; Franklin-Tong et al., 1997; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). This oscillatory behavior is closely associated with growth, and the oscillations in $[Ca^{2+}]_i$ and Ca^{2+} influx at the pollen tube tip have the same periodicity as the pollen tube growth rate (20 to 40 $\mu m\ sec^{-1}$), which also pulses (Pierson et al., 1996; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). This observation has resulted in the suggestion that there is a direct causal link between the two kinds of pulsation, although there remains debate as to whether the changes in $[Ca^{2+}]_i$ drive the oscillations in growth or whether they are caused by them. Data that have been taken to support the former possibility come from experiments demonstrating that increases in Ca^{2+} can trigger the onset of Ca^{2+} oscillations in growth-arrested pollen tubes and that these events can stimulate tip swelling and the resumption of growth (Calder et al., 1997). On the other hand, Holdaway-Clarke et al. (1997) have provided evidence that pollen tube growth rate and the $[Ca^{2+}]_i$ gradient oscillate in phase, whereas pulses in Ca^{2+} influx appear to lag by ~11 sec, implying that Ca^{2+} influx follows growth.

Holdaway-Clarke et al. (1997) have proposed two models that could account for the observed delay between the peak of the $Ca^{2+}{}_i$ gradient and the peak influx of extracellular Ca^{2+} in the apical region. In the first model, they propose that a brief opening of stretch-activated Ca^{2+} channels allows the entry of a small amount of Ca^{2+}. This triggers calcium-induced calcium release from internal stores (e.g., the ER), which increases the $[Ca^{2+}]_i$ gradient. However, there are difficulties with this model, particularly because the ER is not especially concentrated in the tip region and is not closely associated with the plasma membrane (Figure 1; Lancelle and Hepler, 1992). In the second model, which is depicted in Figure 3 and is discussed below, it is suggested that the cell wall acts as a Ca^{2+} store and that periodic changes in the ion binding properties of the pectin in the cell wall could regulate the rate of Ca^{2+} influx, resulting in the observed oscillations (Holdaway-Clarke et al., 1997). Although these models are speculative at present, they do provide hypotheses to test.

What Is the Function of the Apical $[Ca^{2+}]_i$ Gradient?

The precise function of the apical $[Ca^{2+}]_i$ gradient remains unclear, although it seems likely that it is involved in the regulation of vesicle secretion in the apical dome of the pollen tube. It is known that high levels of Ca^{2+} are associated with secretion (Blackbourn and Battey, 1993) and that annexins, which are Ca^{2+} and phospholipid binding proteins that play an important function in secretion, are localized in the apical dome of the pollen tube tip (Blackbourn et al., 1992; Battey

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Figure 2. (continued).

$[Ca^{2+}]_i$ gradient. Note that the basal $Ca^{2+}{}_i$ levels for both of these pollen tubes are very similar. Reproduced from Franklin-Tong et al. (1997), with the permission of Blackwell Science.

(B) Growing pollen tubes exhibit oscillations in $[Ca^{2+}]_i$ at the tip. Ratio imaging of $[Ca^{2+}]_i$ in a growing pollen tube of *Lilium longiflorum* microinjected with fura-2 dextran. Time, in seconds, is given on each image. Note that the apical $[Ca^{2+}]_i$ gradient is not constant but oscillates over time (Holdaway-Clarke et al., 1997).

(C) Reorientation of an *Agapanthus* pollen tube. Calcium Green-1 was microinjected into a pollen tube of *A. umbellatus* together with caged Ca^{2+} (Nitr-5). $[Ca^{2+}]_i$ was imaged using laser confocal scanning microscopy (LCSM) before treatment (first two images) and after release of caged Ca^{2+} in a region to the left of the pollen tube tip (indicated by the arrow). Increases in $[Ca^{2+}]_i$ in the tip region are detected coincident with the reorientation of pollen tube growth over time (given in seconds toward the top of each image). Based on a figure originally published in Malhó and Trewavas (1996). Bar = 10 μm.

(D) Ca^{2+} wave propagated in a *P. rhoeas* pollen tube after release of caged $Ins(1,4,5)P_3$. Calcium Green-1 was microinjected together with caged $Ins(1,4,5)P_3$, and $Ca^{2+}{}_i$ was imaged in a *P. rhoeas* pollen tube by using LSCM. The top image shows the pollen tube before treatment, after which caged $Ins(1,4,5)P_3$ was released throughout the imaged region by UV photolysis (indicated by the arrow). Note the slow increases in $[Ca^{2+}]_i$, which "flood" toward the pollen tube tip in the form of a Ca^{2+} wave following $Ins(1,4,5)P_3$ release (Franklin-Tong et al., 1996). Elapsed time is given in seconds (s) to the right of each image.

(E) Ratio imaging of *P. rhoeas* pollen tubes during the SI response. Fura-2 dextran was microinjected into two pollen tubes to visualize alterations in $[Ca^{2+}]_i$ in response to the addition of incompatible stigmatic S proteins over time (indicated in seconds [s] after the addition). (i) Alterations in $[Ca^{2+}]_i$ in the apical and subapical region of a pollen tube. The oscillating tip-focused gradient is lost within 60 sec. In contrast, $[Ca^{2+}]_i$ in the subapical region continues to increase. (ii) Changes in $[Ca^{2+}]_i$ appear to originate in the shank of the pollen tube, in a region ~100 μm behind the tip. Within 1 to 2 sec (indicated as 0 s), $[Ca^{2+}]_i$ increases from basal levels to ~300 nM, and localized regions rapidly reach 1 μM Ca^{2+}. $[Ca^{2+}]_i$ redistribution occurs and is especially notable at 80 and 100 sec; these changes appear to take the form of a Ca^{2+} wave. Reproduced from Franklin-Tong et al. (1997), with the permission of Blackwell Science.

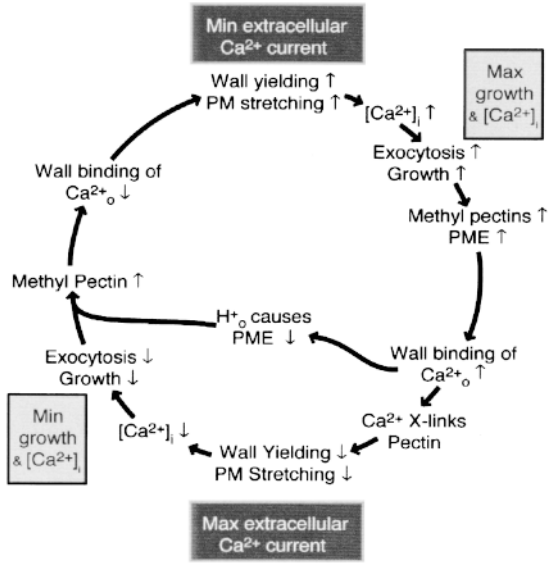

Figure 3. The External Stores Model for Oscillatory Growth in Pollen Tubes.

This model seeks to explain the oscillatory nature of pollen tube growth, which is out of phase with Ca^{2+} influx. See text for details. Ca^{2+}_o, extracellular Ca^{2+}; Max, maximum; Min, minimum; PM, plasma membrane; PME, pectin methylesterase; X-links, cross-links; up and down arrows indicate increase and decrease, respectively. Reproduced from Holdaway-Clarke et al., 1997.

and Blackbourn, 1993; Battey et al., 1999, in this issue). Elevated $[Ca^{2+}]_i$ at the tip is predicted to promote vesicle exocytosis and hence cell elongation. It is therefore tempting to speculate that the colocalization of high Ca^{2+} and high annexin at the pollen tube apex may represent a functional and possibly direct relationship among annexins, Ca^{2+} influx, and vesicle fusion, all of which are required for pollen tube tip extension. Similarly, because microfilament assembly and disassembly are known to be regulated by Ca^{2+} and Ca^{2+}-dependent enzymes, it is also considered likely that the pollen tube cytoskeleton is directly or indirectly influenced by fluctuations in the apical Ca^{2+} gradient.

The Ca^{2+}–pectin model mentioned above (Figure 3; Holdaway-Clarke et al., 1997) has the attractive feature that it attempts to explain oscillatory growth directly in terms of the consequences of the action of high $[Ca^{2+}]_i$. These authors hypothesize that pectin methylesterases (PMEs), which are responsible for cross-linking unesterified pectins, control the rate of production of cell wall binding sites for Ca^{2+}. As shown in Figure 3, when incoming Ca^{2+} binds to the wall, the binding of Ca^{2+} also releases H^+. The resulting acidification could deactivate PME activity, and assuming that exocytosis continues, unesterified methylpectin would be pushed into the tip zone and would stretch the plasma membrane here, leading to tip extension. This could also result in the opening of stretch-activated Ca^{2+} channels and the entry of Ca^{2+} ions at the tip. Such a system would be self-regulating, because incoming Ca^{2+} would be sequestered into the cell wall, the $[Ca^{2+}]_i$ gradient would decrease, and the free Ca^{2+} in the cell wall would result in the cross-linking of pectins, which would slow down exocytosis and growth.

Support for this model comes from studies demonstrating that the pulsing growth of pollen tubes can be accompanied by the deposition of distinct cell wall bands. Wall material deposited during periods of slow growth are enriched with acidic pectins and arabinogalactan proteins and contain relatively less esterified pectin (Li et al., 1992, 1994; Geitmann et al., 1995). Although there is now general acceptance that tip-focused $[Ca^{2+}]_i$ oscillations in pollen tubes are likely to be a general phenomenon, the relationship between Ca^{2+} influx and growth needs further clarification, as does the exact function of the Ca^{2+} gradient.

Which Way to Go? Ca^{2+} Is Involved in the Reorientation of Growing Pollen Tubes

How the perception of extracellular signals influences the modulation of growth patterns and the directionality of plant cells is currently a key question in the plant signaling field. Although the study of “normal” pollen tube growth has provided important information in recent years about the mechanisms that control pollen tube growth, investigations of the signals that may alter growth are also beginning to provide insights. In particular, by studying the inhibition and reorientation of pollen tubes, some of the signaling pathways that modulate pollen tube growth have been identified. Below, I discuss some of the evidence that supports the involvement of Ca^{2+} signaling in modulating pollen tube growth in response to known stimuli.

Pollen tubes undergo necessary changes in direction during their progress through the pistil before they effect fertilization. Indeed, the nature of the signals, signaling pathways, and the mechanisms involved in the reorientation of pollen tube growth are of central importance to an understanding of this process. A series of studies by Malhó (Malhó et al., 1994, 1995; Malhó and Trewavas, 1996) using Ca^{2+} imaging of *Agapanthus* pollen tubes has begun to advance our knowledge on this front. These studies have established a role for $[Ca^{2+}]_i$ in the reorientation of *Agapanthus* pollen tubes. For example, Figure 2C shows the effect on pollen tube orientation provoked by releasing caged Ca^{2+}. Local increases in $[Ca^{2+}]_i$ apparently perturb the polarity of the pollen tube; growth stops temporarily (the apical Ca^{2+} gradient is lost), and the tip swells. Tip swelling coincides with the establishment of a new apical Ca^{2+} gradient and with the resumption of growth, which usually occurs in a new direction (Figure 2C; Malhó et al., 1994, 1995). On the question of how the direction of reorientation may be controlled, Malhó and Trewavas (1996) have shown that the direction and angle of reorientation could be predicted accurately by locally

increasing $[Ca^{2+}]_i$ within the pollen tube, as shown in Figure 2C, and also by locally altering external Ca^{2+}.

These data strongly suggest that Ca^{2+} channel activity in the apical dome plays a critical role in determining pollen tube reorientation. Pierson et al. (1996) have suggested that there is a region of higher Ca^{2+} channel activity within the apical dome that defines the precise point from which elongation will proceed. Malhó and Trewavas (1996) have shown that Ca^{2+} channel activity is localized to the first 20 μm of the pollen tube and that alterations in the exact localization of this activity can influence directional growth in pollen tubes. The nature of the type of Ca^{2+} channels, as mentioned earlier, awaits further investigation. Together, these data suggest that a Ca^{2+}-based signal transduction pathway can control the direction of pollen tube growth. However, it should be borne in mind that there are likely to be many other factors controlling directional growth of the pollen through the pistil tissues toward the ovary. Some of these factors are discussed later.

A Role for Phosphatidylinositol-(1,4,5)-Triphosphate ($Ins[1,4,5]P_3$) in the Regulation of $[Ca^{2+}]_i$ in Pollen Tubes

So far I have discussed the central role for $[Ca^{2+}]_i$ in modulating pollen tube growth and the potential roles of the cell wall and the ER as Ca^{2+} pools. Nevertheless, the signals involved in Ca^{2+} release remain, for the most part, unidentified. However, there is emerging evidence that a functional phosphoinositide signal transducing system operates in growing pollen tubes and that this pathway may be involved in coordinating and regulating pollen tube growth.

The presence of phosphoinositides and phosphatidylinositol phospholipase C activity in pollen tubes of *Lilium longiflorum* was demonstrated some years ago (Helsper et al., 1987). This finding was recently confirmed when it was demonstrated that a Ca^{2+}-dependent $Ins(4,5)P_2$-specific phospholipase C activity is present in pollen of *Papaver rhoeas* (Franklin-Tong et al., 1996).

Further evidence comes from data showing that changes in intracellular concentrations of $Ins(1,4,5)P_3$, as illustrated in Figure 2D, can stimulate large increases in $[Ca^{2+}]_i$ in growing pollen tubes, which initiate in the "nuclear region" and are propagated toward the pollen tube tip (Franklin-Tong et al., 1996). It has been suggested that these waves are mediated, at least in part, by $Ins(1,4,5)P_3$-induced Ca^{2+} release, and a model describing this possibility proposes that the waves are a result of a series of $Ins(1,4,5)P_3$-generating and Ca^{2+}-mobilizing reactions (Franklin-Tong et al., 1996). Although there is good evidence for the presence of $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores in the shank of pollen tubes (Franklin-Tong et al., 1996; Malhó, 1998), their nature remains to be established.

That a functional phosphoinositide signal transducing system involving $Ins(1,4,5)P_3$-stimulated increases in $[Ca^{2+}]_i$ plays a role in both reorientation and inhibition of pollen tube growth is suggested by data showing that changes in intracellular concentrations of $Ins(1,4,5)P_3$ provoke many of the changes in pollen tube tip morphology and growth that were described earlier. One could envisage a two-tier level of control involving phosphoinositide-mediated signals modulating pollen tube growth. In addition to a role for $Ins(1,4,5)P_3$-stimulated increases in $[Ca^{2+}]_i$ in inhibiting pollen tube growth, low-level phosphoinositide turnover appears to be required for normal pollen tube growth (Franklin-Tong et al., 1996).

The SI Response in *P. rhoeas* Pollen Is Mediated by Ca^{2+}

Further evidence for the central role of $[Ca^{2+}]_i$ in modulating pollen tube growth also comes from studies of the SI response in *P. rhoeas*, in which inhibition of "self" pollen in response to a precise, defined signal has been studied in some detail. Several alleles of the stigmatic *S* gene have been cloned, and both stigmatic extracts and recombinant S proteins have been shown to possess the expected *S* allele–specific biological activity, that is, they inhibit incompatible pollen soon after germination (Foote et al., 1994; Franklin et al., 1995; Walker et al., 1996). The current model for the initial events in the SI response proposes that the stigmatic S proteins, which act as signal molecules, bind a membrane-bound pollen receptor in an *S* allele–specific manner, triggering a Ca^{2+}-dependent signal transduction pathway.

Calcium imaging of pollen tubes challenged with S proteins has provided good evidence that Ca^{2+} acts as a second messenger responsible for mediating inhibition of pollen tube growth, usually soon after germination, during the SI response (Franklin-Tong et al., 1993, 1995, 1997). This point is illustrated in Figure 2E. Transient increases in $[Ca^{2+}]_i$, which are triggered virtually immediately and last several minutes, can be detected only when biologically active S proteins are used in combination with incompatible pollen.

What is the effect of these increases in $[Ca^{2+}]_i$? Data from several different experiments suggest that increases in $[Ca^{2+}]_i$ can inhibit pollen tube growth and that the S proteins act as signal molecules and achieve their effect by stimulating alterations in $[Ca^{2+}]_i$ (Franklin-Tong et al., 1993, 1995, 1997). For example, early imaging studies of pollen tubes responding to the SI reaction suggested the *S* allele–specific $[Ca^{2+}]_i$ increases were localized in the shank of the pollen tube (Franklin-Tong et al., 1993, 1995; Drøbak et al., 1997). More recent studies using ratiometric Ca^{2+} imaging have allowed alterations in $[Ca^{2+}]_i$ to be quantified. These studies have provided further evidence that Ca^{2+} waves are elicited by the SI response (Franklin-Tong et al., 1997) and that increases in $[Ca^{2+}]_i$ are detected in the subapical and shank regions of the pollen tube, as shown in Figure 2E. Unexpectedly, after some fluctuation, the tip-focused gradient at the pollen tube tip diminishes to basal levels within ~1 min (Franklin-Tong et al., 1997). This suggests that, whereas

alterations in $[Ca^{2+}]_i$ in the apical region are crucial for some processes regulating pollen tube growth, alterations in $[Ca^{2+}]_i$ in other regions of the pollen tube also play a role. The mechanisms involved, the source of the Ca^{2+}, and the nature of the Ca^{2+} waves are, at present, not known and require further investigation.

The rapidity of the SI response suggests that Ca^{2+} is acting as a second messenger and that increases in $[Ca^{2+}]_i$ are likely to be one of the first events in the signaling pathway. Ca^{2+}-dependent increases in protein phosphorylation triggered by the SI response, which are likely to be downstream of these initial Ca^{2+} signals, have been identified (Rudd et al., 1996, 1997). The increased phosphorylation of a 26-kD cytosolic protein, p26.1, specifically induced by the SI response, appears to be Ca^{2+} and calmodulin dependent (Rudd et al., 1996). This suggests that a Ca^{2+}-dependent protein kinase requiring calmodulin-like domains is activated during the SI response and that it acts as an intracellular signal mediating the SI response in *P. rhoeas* pollen. These observations provide compelling support for the idea that the S proteins profoundly modulate Ca^{2+} homeostasis.

As an indication of the potential complexity of this signaling pathway, p68, another S-specific phosphorylation-responsive pollen protein, has been identified (Rudd et al., 1997). The timing of p68 phosphorylation suggests that its phosphorylation is downstream of p26.1. Surprisingly, however, the kinase(s) responsible for the phosphorylation of p68 is not Ca^{2+} dependent. This suggests that a "second wave" of Ca^{2+}-independent signaling may follow the initial Ca^{2+}-dependent SI signaling.

IT ISN'T ALL Ca^{2+}—OTHER SIGNALING COMPONENTS THAT MAY REGULATE POLLEN TUBE GROWTH

Protein Kinases in Pollen

Although the evidence for signaling, and Ca^{2+}-dependent signaling in particular, in pollen–pistil interactions is now good, there are remarkably little data relating to the activities of protein kinases in pollen. However, as the above discussion illustrates, it is becoming apparent that Ca^{2+}-mediated signaling cascades involving pollen protein kinases are likely to play a major role during pollination. Ca^{2+}-dependent protein kinases were first identified in germinated pollen of *Nicotiana alata* (Polya et al., 1986). Since then, a pollen-specific, Ca^{2+}-dependent, calmodulin-independent protein kinase (CDPK), which is thought to be required for pollen germination and growth, has been cloned from maize pollen (Estruch et al., 1994). This CDPK may be involved in altering the cytoskeletal dynamics required for pollen tube growth.

More recently, soluble and microsomal Ca^{2+}-dependent protein kinases, which share characteristics of CDPKs, have been identified in *N. alata* (Kunz et al., 1996). One of these, Nak-1, has been shown to phosphorylate the stylar S-RNases involved in SI in *N. alata.* Alterations in the phosphorylation status of pollen proteins during incompatible and compatible pollinations have also been reported in rye and in the Brassicas (Wehling et al., 1994; Hiscock et al., 1995). Recent data suggest that Ca^{2+}-dependent protein kinase activity in pollen tubes may be localized in the apical region (Moutinho et al., 1998).

A number of receptor-like protein kinases, including PRK1, LePRK1, and LePRK2, have been cloned from pollen (Mu et al., 1994; Muschietti et al., 1998). These are all functional protein kinases, and although their biological function has not been determined, it has been suggested that PRK1 may have a role in pollen development (Mu et al., 1994), whereas LePRK1 and LePRK2 appear more likely to play roles in pollen–pistil interactions (Muschietti et al., 1998). The existence of functional protein kinases with an extracellular receptor domain clearly provides good evidence that signals are transduced into the pollen grain or tube, presumably from the pistil. The next challenge will be to identify and characterize in detail the pistil components with which the pollen interacts, the nature of the signaling pathways triggered by the interaction, and the resulting responses.

Rho and Small GTP Binding Proteins

The Rho family of GTPases function as key molecular switches, controlling a variety of actin-dependent cellular processes in diverse eukaryotic organisms. Recent work provides good evidence that Rho-type GTPases play a key role in the control of polarized growth of pollen tubes (Lin et al., 1996; Lin and Yang, 1997). For example, a Rho-type GTPase, Rop1, is expressed predominantly in pollen and pollen tubes (Lin et al., 1996), and its localization in a tip–base gradient concentrated toward the apical region implies a role in tip growth and movement of the generative cell in pollen tubes. Moreover, because it is also distributed toward the periphery of the generative cell, where it colocalizes with myosin, Rop GTPase may have a role in the modulation of an actinomyosin motor system involved in the movement of the generative cell (Lin et al., 1996).

Further evidence that Rop may be involved in controlling tip growth comes from elegant functional studies in which microinjected anti-Rop antibodies inhibited pollen tube growth (Lin and Yang, 1997). Not only did these studies provide the first direct demonstration that microinjected antibodies can be used as tools to study pollen tube growth, they also provided good evidence that Rop may regulate a Ca^{2+}-dependent pathway involved in vesicle docking or fusion, whereas a distinct Rho GTPase may mediate cytoplasmic streaming. Further studies aimed at unraveling the signaling pathways and the structural elements with which they interact should greatly enhance our understanding of the apparently complex processes involved in the modulation of pollen tube tip growth.

POLLEN–PISTIL INTERACTIONS: HOW IS POLLEN TUBE GUIDANCE CONTROLLED IN VIVO?

So far I have discussed pollen tube growth in isolation. Although it is clear that pollen can grow without the presence of a stigma or style, there is considerable evidence for interactions between pollen tubes and the transmitting tract of the style, which contains an extracellular matrix of sugars, polysaccharides, glycosylated proteins, and lipids (Lord and Sanders, 1992; Sanders and Lord, 1992), through which they grow. Stylar components are thought to have several functions with respect to pollen tube growth, including adhesion, nutrition, directional guidance, and signaling. Recent data suggest that specific triacylglycerides play a role in pollen tube penetration of tissues by regulating water uptake in a manner that can steer directional growth (Wolters-Arts et al., 1998).

Arabinogalactan proteins (AGPs), which are heavily glycosylated, represent another class of molecule that has been implicated in regulating pollen tube growth through the style (reviewed in Sommer-Knudsen et al., 1997). There is evidence that at least some stylar AGPs are taken up and incorporated into pollen tubes growing in vivo (Lind et al., 1996), and some AGPs, such as tobacco transmitting tissue glycoprotein (TTS), can stimulate pollen tube growth (Cheung, 1995, 1996). Further evidence for the role of TTS comes from the poor growth of pollen on transgenic plants expressing antisense TTS (Wu et al., 1995). Several models explaining how gradients of TTS in the style may help guide pollen tubes to the ovary have been recently reviewed by Cheung (1996). However, the topic of pollen tube guidance is controversial, and recent data (Sommer-Knudsen et al., 1998) suggest that the story may not be quite so clear cut.

POLLEN TUBE–OVULE INTERACTIONS AND FERTILIZATION

Pollen tubes reaching the bottom of the pistil and the ovaries must utilize a complex guidance system as they twist and turn to gain entrance to the ovules through the micropyles. It is currently thought that chemotropic signals play a major role in guiding pollen tubes toward the ovules at this stage. Genetic and biochemical analyses of mutants that exhibit defective directional pollen tube growth in ovary tissue (e.g., Hülskamp et al., 1995b) should help identify some of the factors that mediate pollen tube guidance toward the end of its journey.

Early events in fertilization are at present poorly understood, and it is beyond the scope of this article to describe this process. Nevertheless, this is an active area of research, and recently, by using isolated sperm and egg cells, evidence for a transient increase in $[Ca^{2+}]_i$ upon fertilization and endosperm development after fusion in vitro has been obtained (Digonnet et al., 1997; Kranz et al., 1998). These studies open the way for identifying the signals that mediate early events in fertilization.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Considerable progress has been made during the last 10 years with respect to our understanding of many of the processes involved in the regulation of pollen tube growth. It is now clear that pollen tube growth, which is central to the vital process of sexual reproduction in plants, is tightly regulated. Alterations in $[Ca^{2+}]_i$ have the potential to affect many cellular processes, including vesicle fusion, cytoplasmic streaming, and the cytoskeleton. Although we now know that alterations in $[Ca^{2+}]_i$ are indisputably involved in modulation of pollen tube growth, the nature of these signals, how they are integrated, and the components upon which they act are still largely unknown. The next major challenge will be to identify the major components that interface with the signaling pathways, which will result in a better understanding of the mechanisms involved in the control of pollen tube growth. This is of key importance not only from the fundamental theoretical point of view in expanding our knowledge of how plant cells interact and communicate with each other but also so that, eventually, we can manipulate pollen tube growth for practical purposes in future years.

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