

SPECIAL PAPER

**CALCIUM FUNCTION AND DISTRIBUTION DURING FERTILIZATION  
IN ANGIOSPERMS<sup>1</sup>**

LI LI GE,<sup>2</sup> HUI QIAO TIAN,<sup>2,4</sup> AND SCOTT D. RUSSELL<sup>3</sup>

<sup>2</sup>School of Life Science, Xiamen University, Xiamen 361005, P. R. of China; and <sup>3</sup>Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019 USA

Calcium has an essential signaling, physiological, and regulatory role during sexual reproduction in flowering plants; elevation of calcium amounts is an accurate predictor of plant fertility. Calcium is present in three forms: (1) covalently bound calcium, (2) loosely bound calcium typically associated with fixed and mobile anions (ionic bonding); and (3) cytosolic free calcium—an important secondary messenger in cell signaling. Pollen often requires calcium for germination. Pollen tube elongation typically relies on external calcium stores in the pistil. Calcium establishes polarity of the pollen tube and forms a basis for pulsatory growth. Applying calcium on the tip may alter the axis; thus calcium may have a role in determining the directionality of tube elongation. In the ovary and ovule, an abundance of calcium signals receptivity, provides essential mineral nutrition, and guides the pollen tube in some plants. Calcium patterns in the embryo sac also correspond to synergid receptivity, reflecting programmed cell death in one synergid cell that triggers degeneration and prepares this cell to receive the pollen tube. Male gametes are released in the synergid, and fusion of the gametes requires calcium, according to *in vitro* fertilization studies. Fusion of plant gametes *in vitro* triggers calcium oscillations evident in both the zygote and primary endosperm during double fertilization that are similar to those in animals.

**Key words:** angiosperm sexual reproduction; calcium; cell signaling; double fertilization; gamete interactions; programmed cell death.

When compatible pollen lands on the stigma, pollen grains germinate, forming tubes, which typically elongate through a pollen aperture. The commonly haploid pollen tube continues to elongate within the sporophytic (commonly diploid) tissue of the pistil, proceeding through the style and, once within the ovary, on to the placenta. The tube ultimately reaches an ovule and enters the haploid female gametophyte (commonly known as the embryo sac). The male gametes are released into the embryo sac, are transported to an appropriate location, and then fuse with their respective female target cell where nuclear fusion and fertilization occurs. In this process, complex interactions occur between the male gametic cells, female gametic cells, and the pistil, permitting attraction and precise positioning of the pollen tube in the style, appropriate ovule targeting, and release of male gametes (commonly called sperm cells) inside the embryo sac. Release of the male gametes occurs at a precise location within the embryo sac, facilitating gamete translocation and placing sperm cells in a unique position for gamete recognition. Two male gametes are present within each pollen tube, and each male gamete fuses with a single female target cell. One male gamete fuses with the egg cell, forming the zygote and subsequent embryo lineage, whereas the other male gamete fuses with the female polar nuclei within the central cell, forming the nutritive endosperm.

In the past 30 yr, numerous studies on pollen tube elongation and pollen-pistil interactions have been published. Recent research into sexual reproduction of flowering plants has

increasingly begun to investigate fertilization mechanisms on a biochemical, cell biological, and molecular basis. The role of calcium in this process has received particular attention, as calcium appears to provide a universal signal with pleiotropic effects involving attraction, long and short-distance communication, cellular fusion, and cell signaling. Techniques allowing the isolation and maintenance of pollen and other reproductive cells have been slow to develop because angiosperm gametes are inaccessible and difficult to manipulate. Studies on calcium metabolism in these cells may help us to answer questions about plant fertility and fertilization. This review is among the first to examine the role of calcium in sexual reproduction in flowering plants—a topic that has not been separately reviewed in prior reviews on plant calcium biology.

Calcium is a necessary ion in plant development, with myriad physiological functions. Calcium is tightly compartmentalized in specific states of availability within plants and is required for normal growth and development. In its ionic form, it is known to interact directly and indirectly with calmodulin to regulate other proteins through signal transduction pathways as a second messenger and to serve as a metabolic factor or cofactor in various cellular activities. Calcium mediates cell physiological processes through multiple independent pathways. For many years, calcium regulation in plant physiological processes has been an active topic of research, and results have often been reviewed; some general reviews include Bush (1993, 1995), Gilroy et al. (1993), and Sander et al. (1999). Literature reviews summarizing calcium signaling during pollen tube elongation reflect the particularly active research currently proceeding in this area (Hepler, 1997; Taylor and Hepler, 1997; Zhang and Cass, 1997; Malhó, 1998a; Trewavas and Malhó, 1998; Yang, 1998; Franklin-Tong, 1999a, b; Robinson and Messerli, 2002; McClure and Franklin-Tong, 2006). Among examinations of calcium involvement in sexual

<sup>1</sup> Manuscript received 11 October 2006; revision accepted 8 May 2007.

The authors thank the U.S. Department of Agriculture (NRICGP grant no. 99-37304-8097) and the National Natural Science Foundation of China (grant no. 30670126) for partial support of the research reported in this review.

<sup>4</sup> Author for correspondence (e-mail: hqtian@xmu.edu.cn); phone: +86-592-2186486; fax: +86-592-2181015

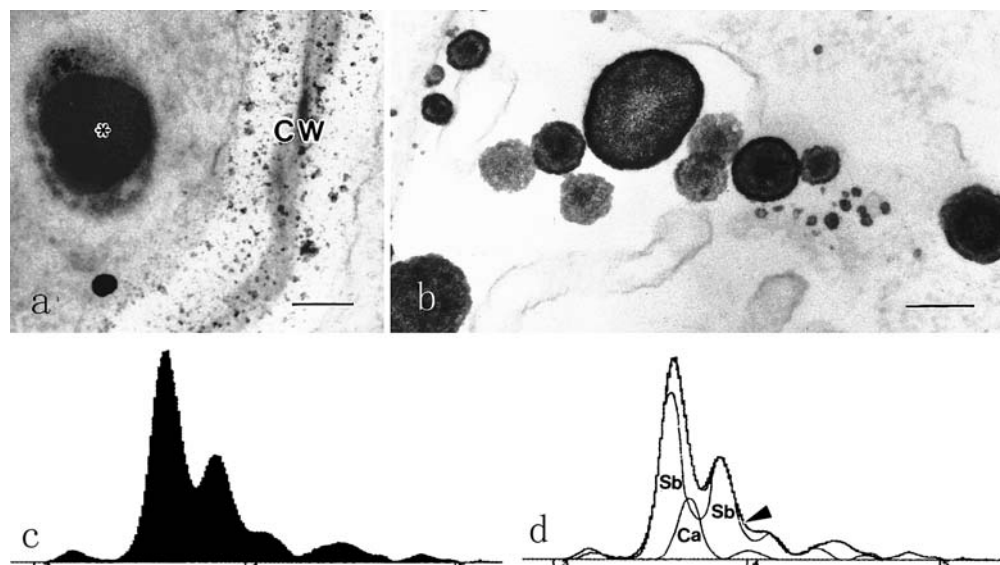


Fig. 1. Transmission electron micrographs of calcium antimonate and corresponding energy-dispersive X-ray spectra from tobacco ovule. Asterisks indicate osmiophilic regions. (a) Nucellus cell containing numerous small calcium antimonate precipitates, particularly in cell wall (cw). Bar = 0.2  $\mu\text{m}$ . (b) Calcium antimonate precipitates in central cell vacuole of tobacco. Bar = 0.2  $\mu\text{m}$ . (c) Energy-dispersive X-ray spectrum composed of overlapping peaks of calcium and antimony in central cell precipitates. (d) Gaussian model of the prominent  $L\alpha$ - and  $L\beta$ -peaks of antimony (Sb) overlapping with the  $K\alpha$ -peak of calcium (Ca); lower lines depict contributions of Sb and Ca in the composite precipitate spectrum. Reproduced from Tian and Russell (1997) with permission of Springer-Verlag.

plant reproduction, however, the study of calcium interactions in the pistil has been relatively neglected. This current review reports on the role of calcium during sexual plant reproduction, including: (1) recent research regarding the function of calcium in pollen tubes and pollen tube guidance; (2) spatial and temporal characteristics of calcium localization in pistils; and (3) general aspects of calcium function during gametic interactions and fertilization in angiosperms. The role of calcium in early plant development is discussed elsewhere (Anil and Rao, 2001).

#### TYPES OF CALCIUM IN CELLS

Calcium in cells exists in several states. The total calcium of a cell is generally purported to range between 0.1 and 10 mM (Nagai et al., 1975; Spanswick and Williams, 1965) and can be divided into free calcium, bound calcium, and stored (loosely-bound) calcium. Free calcium is located in the cytosol, is freely soluble, and is present at concentrations of about 200 nM (Brinley et al., 1977; Bush, 1995; Sanders et al., 1999). Bound calcium has a stronger affinity with macromolecules, which often means that it forms covalent chemical bonds requiring substantial energy to break. Such tightly bound calcium cannot be precipitated by competition with low affinity anions, such as antimonate. This tightly bound calcium can be detected by a wide range of non-specific methods, including X-ray analysis (Chandler and Battersby, 1976; Van Iren et al., 1979) and proton-induced X-ray emission (PIXE, Reiss et al., 1983) (Fig. 1). The highest concentration of bound calcium is found in common crystalline forms, such as calcium oxalate. Loosely-bound calcium is the prevalent form of calcium stored in most cells. This form of calcium is typically present at concentrations above  $10^{-6}$  M and is in dynamic equilibrium with the free

ionized form,  $\text{Ca}^{2+}$ . Stored calcium is often sequestered in the cell wall, localized in specific cellular organelles, or is associated with proteins that use calcium as a coenzyme or that control calcium concentration. When physiological conditions in the cell alter the concentration of  $\text{Ca}^{2+}$ , the solubility coefficient ( $K_{sp}$ ) predicts the highest concentration of ionic free calcium that can accumulate before it spontaneously begins forming ionic bonds. If  $\text{Ca}^{2+}$  exceeds the  $K_{sp}$  for a given calcium-binding molecule, then excess calcium is bound; however, if  $\text{Ca}^{2+}$  falls below the  $K_{sp}$ ,  $\text{Ca}^{2+}$  is dynamically released from its ionically bound state. Thus, high concentrations of free  $\text{Ca}^{2+}$  can be dynamically depleted above the  $K_{sp}$  by reversible ionic binding, effectively controlling the free calcium concentration. The effective  $K_{sp}$  of the binding molecule, which is often a protein, may be dramatically altered in binding and functionality by changes in molecular or ionization state, including those induced by pH and other environmental factors. Protein alterations through enzymatic modification, or linkage of control molecules, including phosphorylation, also control calcium binding. Any such changes in ionic state of the molecule may alter its ability to bind calcium. Because they occur in an unbound soluble form, some of these calcium binding proteins can serve as transport molecules, acquiring calcium from storage and releasing it where calcium is required (Tandler et al., 1970).

Each of these forms of calcium has a different physiological function. Changes in free calcium concentrations above a low threshold amount allow free  $\text{Ca}^{2+}$  to act as a second messenger, serving as an early product in signal transduction. Because released free  $\text{Ca}^{2+}$  may trigger additional  $\text{Ca}^{2+}$  pools to be released through activation of ion channels or conversion of bound to free calcium, this phase of cell signaling may result in dramatic amplification of local  $\text{Ca}^{2+}$  levels. Such free calcium release to the cytoplasm and locally amplified calcium

concentrations characterize cell signaling and transmission of external stimuli. Receptors like calmodulin and other calcium-binding proteins may trigger changes in active sites or alter the activity of ion channels, which may produce spikes in free calcium concentration. Examples of such interactions abound in cell biology and include checkpoint release during mitosis or meiosis. Bound calcium also often forms an integral part of the structure of the cell, particularly in such dense crystalline forms as calcium crystals and cell walls. Interestingly, it may also be an early predictor of future programmed cell death, as for instance in the anther ring cells of tobacco, which fuse the locules of anther sacs (Sanders et al., 2005). In the tobacco ring cells, programmed cell death is preceded by the accumulation of abundant fine druses in vacuoles, which may contribute to triggering cell death.

Dynamically sequestered calcium, as mentioned before, is typically ionically rather than covalently bound. This calcium store is dynamic and may therefore be readily converted to (and from) free calcium through interaction with a wide variety of control proteins—in this sense such proteins have calmodulin-like properties. Presumably, a wider examination of expressed transcripts and proteomic data in pollen (Hruba et al., 2005; Sheoran et al., 2006) and other tissues (Swanson et al., 2005) may aid in identifying specific protein candidates for such calcium binding; information about such products is likely to be readily available for bioinformatic data mining in international genome and proteome related databases. To avoid the potentially toxic effects of free  $\text{Ca}^{2+}$ , the concentration of  $\text{Ca}^{2+}$  in the cytoplasm and nucleus in resting cells is three to four lower than in other cellular compartments (Bush, 1993; Gilroy et al., 1993). However, when the cell is stimulated by external stimuli, free calcium is released, calcium channels open, and free  $\text{Ca}^{2+}$  enters through the plasma membrane. Sequestered calcium is also released from the ER, vacuole, and mitochondria (Marmé, 1989; Trewavas and Malhó, 1998). Wick and Hepler (1982) characterized this non-covalently sequestered calcium as loosely bound calcium because of its exchangeable property.

#### CALCIUM EFFECTS ON POLLEN GERMINATION

When pollen grains are germinated in small numbers in vitro, the rates of germination and elongation are often less than in larger scale pollen culture. This population effect can be overcome by adding more pollen, but it may also be overcome by adding more calcium to the medium (Kwack and Brewbaker, 1961). Confirmation of this phenomenon has been obtained in 86 species representing 39 plant families (Brewbaker and Kwack, 1963), so this represents a widespread calcium dependency among flowering plants.

Uptake of calcium by germinating pollen grains has been directly demonstrated using the radioisotope  $^{45}\text{Ca}^{2+}$  (Bednarska, 1989b), which is rapidly incorporated into pollen. Accumulation of calcium in the membranes of germinating pollen is readily illustrated using the fluorescence microscopic probe, chlorotetracycline (CTC) (Polito, 1983). At the onset of pear-pollen hydration, the first faint CTC fluorescence was located at the periphery of the pollen grain, associated with germinal apertures. Following hydration, fluorescence continued to intensify near the pore. Calcium accumulation was also demonstrated in the apertures of lily pollen using similar techniques, reflecting a distinct polarization of calcium (Reiss

and Herth, 1978). One hypothesis for calcium uptake is that pollen hydration may trigger increased turgor that allows uptake through stretch-activated calcium channels (Feijó et al., 1995). This could, in turn, maintain calcium polarization in the pollen grain, amplify polarization of calcium distribution in the pollen tube, and create a sustainable gradient.

During pollen germination, a calcium effect has also been confirmed using calcium antagonists to inhibit germination (Bednarska, 1989b), although pollen grains in some plants can germinate in media without calcium. Presumably, the latter plants have some calcium stores in the pollen wall, which may be released into the medium during pollen hydration (Shivanna and Johri, 1985; Heslop-Harrison, 1987). In rice pollen grains, abundant calcium exists within the pollen grain before germination (Tian et al., 1998).

#### CALCIUM EFFECTS ON POLLEN TUBE ELONGATION

Exogenous calcium in the immediate environment affects both pollen germination and tube growth. A famous assay done by Mascarenhas and Machlis (1962) revealed a chemotropic response of pollen tubes of *Antirrhinum majus* to external calcium gradients in vitro. In cultured pollen tubes of *Tradescantia virginiana*, the optimum concentration for elongation was from  $10^{-4}$  to  $10^{-3}$  mol/L calcium in the medium (Picton and Steer, 1983). In other plants, pollen tubes grew poorly if calcium concentrations in the medium contained higher or lower concentrations (Morré and Vanderwoude, 1974; Reiss and Herth, 1980; Steer and Steer, 1989; Obermeyer and Weisenseel, 1991). In culturing pollinated styles of tobacco, pollen tube growth also required supplemental calcium in the medium (Tian and Russell, 1997b). When the external calcium concentration exceeds  $5 \times 10^{-6}$  M, actin filaments in the pollen tube of *Lilium longiflorum* become fragmented and cytoplasmic streaming stops (Kohno and Shimmen, 1987, 1988). In media containing low concentrations of calcium, generally the pollen tube apex swells, and the cell wall at the tip becomes very thin and discontinuous. In media containing high concentrations of calcium, however, the wall at the tip of the tube becomes overly thickened. Lowering calcium concentrations appears to diminish the elongation of the tube by causing an excessive quantity of vesicles to accumulate at the tip, inducing apical swelling (Morré and Vanderwoude, 1974; Picton and Steer, 1983; Steer and Steer, 1989). High calcium concentrations appear to accelerate vesicle fusion at the pollen tube tip, but cytoskeletal elements may be altered, contributing to a thickened wall at the tube tip.

External effects of calcium on pollen tube growth have been confirmed by adding calcium channel blockers or antagonists into the medium (Bednarska, 1989b; Heslop-Harrison and Heslop-Harrison, 1990). Pollen tube growth is inhibited by experimentally opening calcium channels using the calcium ionophore A23187 in the medium. Ionophore A23187 disturbs the calcium gradients inside and outside the pollen tube, permitting  $\text{Ca}^{2+}$  to pass freely through the plasma membrane (Reiss and Herth, 1978, 1979b; Picton and Steer, 1983; Bednarska, 1989a).

**Calcium distribution in pollen tubes**—Intracellular calcium has been measured using a variety of localization techniques. Each method displays unique sensitivities and weaknesses, and the method by which the probes are applied vary in their

invasiveness and reliability. As different techniques have emerged, they have been applied to accumulate complementary data on the different forms of calcium. Using the radioisotope  $^{45}\text{Ca}^{2+}$  and radioautography, Jaffe et al. (1975) demonstrated that externally absorbed calcium first accumulated at the pollen tube apex. Proton-induced X-ray emission (PIXE), which measures total calcium, indicated that concentrations in pollen tubes of *Lilium longiflorum* range from 1300 ppm calcium at the tip to 800 ppm at the base (Reiss et al., 1983). Chlorotetracycline (CTC) labeling reveals primarily membrane-bound calcium and indicates that membrane-sequestered calcium displays a similar calcium gradient (Reiss and Herth, 1978, 1979a, 1985; Picton and Steer, 1983; Polito, 1983; Bednarska, 1989a). According to ultrastructural examination, this membrane-bound calcium in the pollen tube is mainly localized in the endoplasmic reticulum (Reiss and McConchie, 1988; Cresti and Tiezzi, 1990).

The detection of free calcium is the most difficult, given the mobility of unbound calcium and its ability to dramatically change the cytoplasm at exceedingly low functional concentrations. In animals, transient spatial and temporal changes in the quantity of free calcium ions ( $\text{Ca}^{2+}$ ) are still considered to be among the first detectable events in a signal transduction cascade arising from stimulation of the outside of the cell (Tang et al., 2000; Sanders et al., 2002). In that case, the binding of ligand and receptor evokes an internal release of free calcium that serves as a potent second messenger triggering numerous downstream effects. In pollen tubes of *Lilium longiflorum*, concentrations of free calcium estimated using quin-2 fluorescence are reported as  $10^{-7}$  mol/L near the tip and  $2 \times 10^{-8}$  mol/L at the base of the tube (Nobiling and Reiss, 1987). Free  $\text{Ca}^{2+}$  concentrations measured using the fura-2 fluorescence indicator were about 10 times lower, with calcium concentrations of 1.7 to 2.6  $\mu\text{mol/L}$  at the tip and 60 to 100 nmol/L at 100  $\mu\text{m}$  behind the tip (Obermeyer and Weisenseel, 1991). Fura-2 dextran, which is a membrane-impermeable injected label, provided  $\text{Ca}^{2+}$  measurements that were nearly one-third lower at the tube tip (490 nmol/L at the extreme tip) and 170 nmol/L at 10–20  $\mu\text{m}$  behind the tip. The free-calcium gradient reportedly drops sharply within 10–20  $\mu\text{m}$  of the tube tip (Miller et al., 1992). Although values of free calcium in these reports differ markedly, a free-calcium gradient appears consistently regardless of technique. Several more recent reports have indicated that the  $\text{Ca}^{2+}$  gradient is restricted to just the apical 20  $\mu\text{m}$  of the pollen tube tip. Changes in free calcium beyond the first 20  $\mu\text{m}$  of the pollen tube were significantly lower (Pierson et al., 1994, 1996; Malhó and Trewavas, 1996; Franklin-Tong et al., 1997; Hepler, 1997; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). The most modern approach uses the recombinant yellowameleon calcium reporter. This technique avoids the loading problems of the former free calcium probes and is temporally sensitive enough to detect fluctuations in the calcium gradient of the pollen tube tip. Also, its protein reporter can be targeted using specific peptide additions to selected subcellular compartments (Watahiki et al., 2004).

Interestingly, the  $\text{Ca}^{2+}$  gradient exists only in growing pollen tubes. Upon death or the addition of calcium channel blockers or antagonists into the medium, the calcium gradient disappears from the tip of the pollen tube, and the tube stops elongating. When such arrested tubes are transferred to a medium without calcium blockers or antagonists, they rapidly reestablish a  $\text{Ca}^{2+}$  gradient and reinitiate elongation. Strongly concentrated  $\text{Ca}^{2+}$

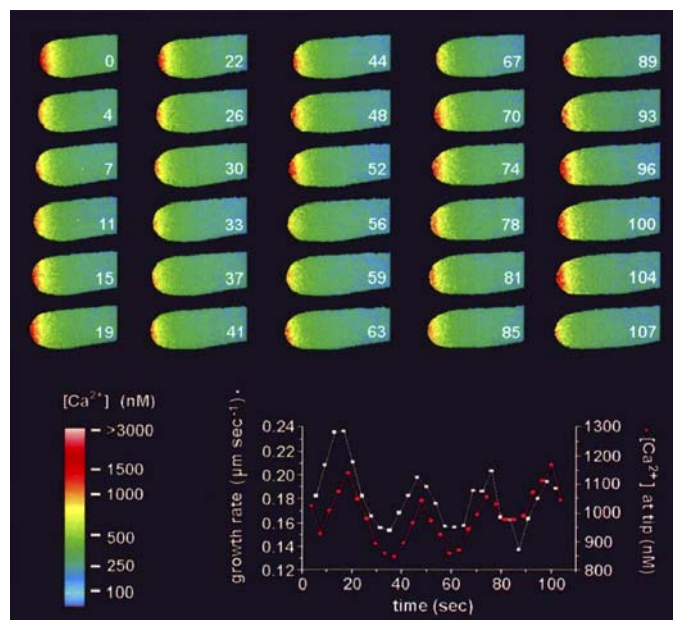


Fig. 2. Pseudocolor ratio imaging of elongating *Lilium longiflorum* pollen tubes loaded with fura-2-dextran displays conspicuous oscillations of apical  $[\text{Ca}^{2+}]$ ; elapsed time in seconds is superimposed on corresponding images. Graph (below) depicts pollen tube elongation rate (in white) vs. simultaneously measured  $\text{Ca}^{2+}$  concentration at the tip (in red). Reproduced from Holdaway-Clarke et al. (1997) with permission of P. K. Hepler (University of Massachusetts, Amherst, MA, USA).

gradients in the pollen tube tip closely correspond with vigorous tip elongation (Rathore et al., 1991; Obermeyer and Weisenseel, 1991; Miller et al., 1992; Li et al., 1994; Malhó et al., 1994, 1995; Pierson et al., 1994; Feijó et al., 1995).

**Oscillation of the calcium gradient and pulsatory growth of the pollen tube**—Pierson et al. (1994) used more rapid, ratiometric calculations with a confocal microscope and confirmed the steepness and depth of the tip-focused intracellular  $\text{Ca}^{2+}$  gradient of lily pollen tubes, which varied from  $>3.0 \mu\text{M}$  at the apex to  $\sim 0.2 \mu\text{M}$  within 20  $\mu\text{m}$  from the tip. Although levels of intracellular  $\text{Ca}^{2+}$  were assumed to remain essentially stable over time, further examination revealed that, based on measurements using a  $\text{Ca}^{2+}$ -specific vibrating electrode, extracellular calcium entered the pollen tube at influx rates between 1.4 and 14  $\text{pmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ . These rates of extracellular calcium influx into the pollen tube were uneven but reasonably periodic. The tubes elongated in pulses that corresponded with periodic deposition of cell wall components (Pierson et al., 1995). Ratiometric ion imaging of the intracellular  $\text{Ca}^{2+}$  gradient indicated that the high point of the gradient fluctuated in magnitude from 0.75 to more than 3  $\mu\text{M}$  during  $\sim 60$ -s measuring intervals. Elevation of the calcium gradient appeared to be correlated with an increased rate of tube growth (Pierson et al., 1996). Holdaway-Clarke et al. (1997) confirmed that the tip-focused  $\text{Ca}^{2+}$  gradient oscillates with the same periodicity as pollen tube growth, but learned that the pulses are slightly out of phase. The extracellular calcium influx is delayed by about 11 s compared with the oscillation of the  $\text{Ca}^{2+}$  gradient (Fig. 2). They therefore inferred that the delay in the extracellular inward

current may be related to the cyclic depletion of  $\text{Ca}^{2+}$  stores in the pollen tube immediately before a similarly out-of-phase cycle of replenishment.

Determining how  $\text{Ca}^{2+}$  oscillations, tube pulsatory growth, and extracellular calcium influxes are related represents a significant recent advance in understanding the extremely dynamic nature of calcium in pollen tube elongation (Malhó et al., 1998; Trewavas, 1999). Pulsatory tube elongation, however, is not required, even in plants that characteristically display pulsatory pollen tube behavior. Geitmann and Cresti (1998) found that inorganic  $\text{Ca}^{2+}$  channel inhibitors  $\text{La}^{3+}$  and  $\text{Gd}^{3+}$  could trigger the pulsating pollen tubes to abandon their rhythm and continue to elongate with steady rates. Organic inhibitors of  $\text{Ca}^{2+}$  channels, nifedipine and verapamil, slowed pulse frequency but did not block pulses. These results strongly suggest that at least two types of  $\text{Ca}^{2+}$  channels occur in the plasma membrane of the tube tip. If apical elongation of the pollen tube is inhibited, periodic membrane traffic may still occur with nearly the same periodicity as was exhibited during normal tube elongation (Parton et al., 2003).

Pulsatory exocytosis and cyclic  $\text{Ca}^{2+}$  correspond well with pulsatory patterns of pollen tube elongation, but it is becoming increasingly evident that models of oscillatory pollen tube elongation will not be explained only by cyclic cell wall loosening under turgor (Messerli and Robinson, 2003). Cellular oscillations apparently involve an integration of plasma membrane ion fluxes that constitutes a "cytosolic choreography" of proton and calcium fluxes that operates in concert with intake fluxes of anions, including potassium and chloride (Feijó et al., 2001; Zonia et al., 2001). Chloride efflux at the pollen tube apex is in turn affected by fluxes in inositol 3,4,5,6-tetrakisphosphate with momentary interruptions of chloride efflux associated with elongation maxima (Zonia et al., 2002). Pollen tube behavior is also influenced by other ions, notably  $\text{K}^+$ , which appears to be controlled by gated channels (Fan et al., 2001; Mouline et al., 2002). Increasing information is available concerning the involvement of Rop GTPase-dependent regulatory proteins (Fu et al., 2001; Baxter-Burrell et al., 2002; Gu et al., 2003), Rop1ps (Zhao and Ren, 2006), Rop2 GTPases (Jones et al., 2002), and Rac-like GTPases (Cheung et al., 2003) in the control of endo- and exocytosis in the pollen tube apex (Camacho and Malhó, 2003).

Dynamic calcium concentrations play a central role in increasingly complicated models of growth oscillations in pollen tubes, both in a direct physiological role and more importantly in signaling (Holdaway-Clarke et al., 2003). Fluctuations in the pollen tube tip-focused calcium gradient are not reflected in nuclear calcium levels according to analysis using the recombinant yellowameleon calcium reporter (Watahiki et al., 2004). Clearly, the occurrence of calcium oscillations in pollen tubes has become an important part of our current model for understanding calcium oscillation in higher plant systems (see reviews by Evans et al., 2001; Gradmann, 2001).

#### ROLE OF THE FUNCTIONAL CLASSES OF CALCIUM IN POLLEN TUBE ELONGATION

The three types of calcium in plant cells—bound, loosely bound, and free calcium—although interchangeable, clearly have different functions and use different controlling molecules during pollen tube elongation. These multiple forms of

calcium, their spatial localization, and temporal characteristics compound the complexity of calcium mediation during pollen tube elongation (Hepler, 1997; Malhó, 1998a, b; Yang, 1998; Franklin-Tong, 1999a). The following sections summarize some significant advances in our understanding of calcium regulation in the context of pollen tube elongation.

**Cytosolic free calcium function during pollen tube elongation**—The ability of the pollen tube to reorient tip growth is at the heart of understanding how tubes can so precisely target a single cell within the female embryo sac. Hypotheses that pollen tubes grow toward the ovule by such tropistic mechanisms as chemo-, thigmo- or electrotopism have met with at best equivocal success. What molecular changes would precede and trigger pollen tube curvature? Calcium gradients have long been implicated in regulating pollen tube tip elongation, but their role in determining the orientation of tube growth was not well supported by experimental evidence until the 1990s.

Rui Malhó's group provided evidence that the directionality of *Agapanthus umbellatus* pollen tubes could be modified by iontophoretic introduction of calcium and by weak electrical fields, in which the pollen tubes elongate toward the cathode. Introducing a localized gradient of ionophore A23187, which is believed to open calcium ion channels, causes the pollen tube tip to reorient toward the A23187. When the  $\text{Ca}^{2+}$  channel blocker  $\text{GdCl}_3$  was added to the growth medium, tip elongation became oriented away from  $\text{GdCl}_3$ . The region of highest concentration of cytosolic free calcium was closely aligned with tip reorientation and the direction of future elongation.

A further demonstration of the effect of free calcium was obtained by microinjecting caged  $\text{Ca}^{2+}$  into living pollen tubes. These injected pollen tubes were then eccentrically irradiated with UV light near their tips, causing photolysis of the cages and release of free  $\text{Ca}^{2+}$  within the pollen tube at that location. A resulting transient rise in free  $\text{Ca}^{2+}$  induced a reorientation of tip elongation toward the site of irradiation. Tip growth resumed near the irradiated region and caused a sustained reorientation of tip elongation. The site of tip reorientation corresponded closely with the local release of  $\text{Ca}^{2+}$ . This pattern was reinforced by a decline in  $\text{Ca}^{2+}$  levels on the opposite side of the tube, completing the reorientation (Malhó et al., 1994, 1995; Malhó and Trewavas, 1996). Thus, areas of rich  $\text{Ca}^{2+}$  within a gradient can reorient tip growth and thereby establish the directionality of future pollen tube elongation. A related study demonstrated that a kinase in the pollen tube apex may also be involved in regulating localized  $\text{Ca}^{2+}$  channel activity (Moutinho et al., 1998).

The signal-transduction intermediate inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) can reportedly trigger calcium release from stores in the cell, as well as serve as a second messenger controlling numerous cellular processes (Berridge, 1993). To examine the effect of locally introduced  $\text{InsP}_3$  on pollen tube elongation, Malhó (1998c) microinjected caged  $\text{InsP}_3$  into pollen tubes of *Agapanthus umbellatus*, then photolysed the cages with spot irradiation. Releasing caged  $\text{InsP}_3$  in the apex, subapical, and nuclear regions of pollen tubes resulted in a transient increase of  $\text{Ca}^{2+}$  in these regions. When caged  $\text{InsP}_3$  was released in the nuclear or subapical region, the growth axis of pollen tube was realigned. An undesirable consequence was that photolysis elicited transitory increases in tube elongation, causing tips to burst. These results clearly indicate that calcium is a unique and separate part of the signal transduction pathway controlling

tube guidance. The addition of cAMP, which affects the localized release of  $\text{Ca}^{2+}$ , may reorient tip elongation as well (Moutinho et al., 2001).

**Calcium in pollen tube wall**—Among the primary targets of exogenously provided  $^{45}\text{Ca}^{2+}$  is the pollen tube wall (Brewbaker and Kwack, 1963), where the radioisotope incorporates into carboxyl groups in the pectic components of the cell wall (Kwack, 1967). Calcium associated with pollen tube walls is frequently abundant but rarely is incorporated directly at the tip, because calcium-crosslinked pectin may behave as a rigid and insoluble gel (McNeil et al., 1984). The bipartite pollen tube wall consists of an inner sheath of callose and an outer region of fibrillar layers containing abundant pectin. The tip of the pollen tube is composed of a single pectin layer (Heslop-Harrison, 1987). In immunocytochemical studies, at least two types of pectin were labelled in the pollen tube wall: a polygalacturonic acid form of pectin and methyl-esterified pectin. Acid pectins capable of crosslinking  $\text{Ca}^{2+}$  are localized in the subapical region of the pollen tube. In contrast, richly methyl-esterified pectins are particularly abundant at the pollen tube tip, where they may inhibit  $\text{Ca}^{2+}$  crosslinking. Secretory vesicles in the pollen cytoplasm also appear to contain abundant methyl-esterified pectin (Li et al., 1994; Geitmann et al., 1995; Jauh and Lord, 1996; Geitmann and Parre, 2004). Tube elongation represents a delicate balance between the ability of the tube wall to withstand bursting and the high native osmolality of the pollen cytoplasm (Cresti and Tiezzi, 1992; Feijó et al., 1995). When secretory vesicles release their methyl-esterified pectin at the tube tip, their contents merge to become incorporated into the tube wall. Upon formation, this tip region is the most elastic in the tube and is also likely to be the expansion point under internal turgor pressure. Following tube tip expansion, methyl-esterified pectin migrates basipetally from the tip region. Pectin is deesterified and becomes crosslinked with  $\text{Ca}^{2+}$ , which contributes much to the rigidity and mechanical strength of the pollen wall as well as providing turgor pressure in the other regions of the tube (Li et al., 1994). In vivo, the region of the pollen tube wall adjacent to the micropyle in ovules of tobacco and *Plumbago* contains abundant calcium-induced precipitates, indicating high concentrations of loosely bound calcium (Tian and Russell, 1997a; Tian et al., 2000).

**Other calcium functions during pollen tube growth**—The tip gradient of calcium in the living pollen tube is essential to vesicle secretion at the tube tip. As discussed before, all attempts to alter the gradient also alter the pattern of exocytosis. The elevated concentration of calcium near the secretory membrane appears to be essential for vesicle fusion, as indicated by its involvement in animal as well as plant systems (Almers, 1990; Zorec and Tester, 1992; Battey and Blackburn, 1993). During membrane fusion, the role of  $\text{Ca}^{2+}$  appears to be to draw the docked vesicle membrane into apposition with the plasma membrane by counterbalancing the native negative charge of their constituent phospholipids (Battey et al., 1999). Vesicle secretion at the tip of the pollen tube is an intense, localized form of exocytosis that, perhaps not surprisingly, involves an especially steep gradient of elevated calcium. In pollen tubes, highly active Golgi bodies produce abundant vesicles that migrate to their fusion site via actomyosin associations. The vesicles fuse with the plasma membrane at the tip of the pollen tube, secreting both wall and

plasma membrane material for expansion (Hepler et al., 1994). These areas of elevated  $\text{Ca}^{2+}$  in lily pollen tubes closely resemble conditions in other sites of intense exocytosis (Roy et al., 1999).

Maintenance of the calcium gradient in the tip is characteristic of vigorous pollen tube elongation, but mechanistic details are still not clear. Calcium concentrations outside the pollen need to exceed those at the tip, suggesting that tightly localized calcium channel activity may be sufficient to sustain an exceedingly tight gradient. Even a cursory examination of elongating pollen tubes reveals intense cytoplasmic streaming in the tube, but there is a transition in this pattern of movement from directional to saltatory movement toward the tip (Heslop-Harrison et al., 1991). High concentrations of calcium at the tip induce fragmentation of actin filaments and inhibit cytoplasmic streaming at the extreme tip because of F-actin instability (Kohno and Shimmen, 1987, 1988). The high concentration of calcium within the final 20  $\mu\text{m}$  of cytoplasm at the tube tip appears to create a relatively calm environment for vesicle transport and fusion (Steer and Steer, 1989). The negatively charged vesicles (Heslop-Harrison et al., 1991) may then continue their forward migration through attraction to the positively charged  $\text{Ca}^{2+}$ -rich tip. Although the high calcium concentration inhibits cytoskeleton formation in this region, it promotes the directed transportation of secretory vesicles and their adherence and fusion with the plasma membrane of the tube tip.

Sperm cells in growing pollen tubes are typically located somewhat distant from the tip, for instance  $\sim 90 \mu\text{m}$  from the tip in maize, a location where calcium concentrations are reasonably low (G. Zhang et al., 1997). This condition seems to improve the long-term viability of sperm cells. Maintaining isolated sperm cells requires a suitable concentration of exogenous calcium for membrane integrity (perhaps  $< 1 \text{ mM}$ ), but the concentration is critical or the useful lifespan of viable sperm cells is very brief (G. Zhang et al., 1992, 1995).

#### CALCIUM DISTRIBUTION IN STIGMATIC TISSUE

When a compatible pollen grain lands on the stigma, it germinates and forms a pollen tube with an elongating tip. Using chlorotetracycline (CTC), Tirlapur and Shiggaon (1988) found abundant membrane-bound calcium in papillae of *Ipomoea batatas*. Bednarska (1989c), who also used CTC, found a similar result in *Ruscus aculeatus* L. and confirmed this with X-ray microanalysis, further observing that germinating pollen of *Primula officinalis* and *R. aculeatus* absorb calcium from the stigma (Bednarska, 1991). Studies using potassium antimonate precipitation indicate that loosely bound calcium in sunflower is more abundant on the receptive surface of the stigma, especially outside and inside the papillae, than on nonreceptive surfaces (J. Zhang et al., 1995). Abundant calcium antimonate precipitates are also reported in the intercellular matrix of the stigmatic tissue of cotton (J. Zhang et al., 1997) and on the stigmatic surface of *Brassica oleracea* following pollination, particularly when the pollen grain lands and tube germinates on the stigmatic papillae (Elleman and Dickinson, 1999). The occurrence of abundant calcium in and on stigmatic surfaces is in accord with the need for extracellular calcium for pollen germination and tube elongation in vitro (Brewbaker and Kwack, 1963).



## CALCIUM DISTRIBUTION IN STYLAR TISSUE

After pollen germination, the tubes penetrate the stigmatic surface and grow into the transmitting tissue of the style. Mascarenhas and Machlis (1962) found a chemotropic response of pollen tubes to calcium and observed a gradient of calcium concentrations in pistil tissues of *Antirrhinum majus* proceeding from the stigma to the ovule. Based on in vitro assays, they concluded that the tubes responded to a calcium gradient in the pistil, which causes tubes to elongate toward the embryo sac. Calcium gradients have been confirmed in the pistils of a number of flowering plants, including *Gladiolus gandavensis* (Day et al., 1971), but calcium gradients were absent in several other flowering plants (Glenk et al., 1971; Mascarenhas, 1975). More recent assays using antimonate have confirmed that loosely bound calcium stores occur in the transmitting tissue of a number of plants, including *Brassica napus* (Mao et al., 1992), *Helianthus annuus* L. (J. Zhang et al., 1995), *Gossypium hirsutum* L. (J. Zhang et al., 1997), and *Lilium longiflorum* (Zhao et al., 2004). Calcium-induced antimonate precipitates were mainly localized in the apoplastic system of the transmitting tissue, i.e., in the intercellular matrix and cell wall, where pollen tubes elongate. In contrast, fewer calcium precipitates were localized within the cells of the parenchymatous tissue. The occurrence of abundant calcium in the transmitting tissue suggests that the calcium required for tube elongation in vitro is obtained from the rich calcium environment of the style during in vivo growth. Calcium as a directional cue, however, appears to be effective only in the pollen tubes of a restricted number of plants because numerous flowering plants are insensitive to such a calcium signal (Johnson and Preuss, 2002; Higashiyama et al., 2003).

Calcium is also implicated in the self-incompatibility response of self incompatible (SI) plants. When S-glycoprotein is extracted from self-incompatible stigmas of *Papaver rhoeas* and is introduced into the culture medium, the concentration of calcium increases in tubes, and this increase appears to inhibit tube elongation (Franklin-Tong et al., 1993). However, when either compatible or heat-denatured incompatible stigmatic S-glycoproteins were introduced into the culture medium, no rise in  $\text{Ca}^{2+}$  levels of pollen tubes was detected, and elongation continued normally (Franklin-Tong et al., 1993). Caged  $\text{Ca}^{2+}$  introduced into pollen tubes did not alter tube elongation, but upon cage photolysis, a similar inhibition of tube elongation was observed accompanying artificially elevated internal levels of  $\text{Ca}^{2+}$ . Photoactivation of caged  $\text{InsP}_3$  elicits a similar inhibitory response (Franklin-Tong et al., 1996). The potential effect of contamination by other stylar components was eliminated by cloning the SI-gene and expressing it in *E. coli*; the purified gene product also elicited the same result. Thus, the S-protein alone appears to be sufficient for triggering the  $\text{Ca}^{2+}$  signal during the pollen SI response (Franklin-Tong et al., 1995).

Imaging the calcium directly established that S-protein added to the medium resulted in an influx of extracellular  $\text{Ca}^{2+}$  at the "shank" of the pollen tube. The influx of extracellular  $\text{Ca}^{2+}$  appears to play a role in the *Papaver* SI response (Straatman et al., 2001). The addition of the  $\text{Ca}^{2+}$ -antagonist Verapamil or the calcium channel blocker  $\text{La}^{3+}$  allowed pollen of *Secale* to overcome the self-incompatibility response and permitted elongation into the style (Wehling et al., 1994). Calcium is believed to regulate special transduction reactions during the self-incompatibility response, likely by controlling

the activity of protein kinases.  $\text{Ca}^{2+}$  influx differs between compatible and self-incompatible plants, suggesting an active role for the influx of extracellular  $\text{Ca}^{2+}$  in the SI response (Franklin-Tong et al., 2002). In *Nicotiana glauca*, a plant with gametophytic self-incompatibility, self-pollen tube elongation was inhibited by an S-related ribonuclease in the stylar transmitting tissue (McClure et al., 1990). Presumably, calcium-dependent protein kinases from the pollen tube activated the S-ribonuclease of the transmitting tissue, resulting in the digestion of RNA in the pollen tube and the inhibition of tube growth (Kunz et al., 1996). Immunological studies have indicated the occurrence of calmodulin and calmodulin-like and calreticulin-like proteins that could be involved in  $\text{Ca}^{2+}$ -related cell signaling during pollen-pistil interactions (Lenartowska et al., 2001, 2002; Rudd and Franklin-Tong, 2001, 2003; Snedden and Fromm, 2001).

## CALCIUM DISTRIBUTION IN OVULAR SOMATIC TISSUE

After pollen tubes emerge from the transmitting tissue and enter the ovary, they elongate onto the placental surface within the ovarian locule. As pollen tubes travel along the placental surface toward the proximal end of the ovary, individual pollen tubes diverge from the masses by  $90^\circ$  before elongating through the last several micrometers to enter the ovular micropyle. In all plants investigated to date (pearl millet, Chaubal and Reger, 1992a; sunflower, J. Zhang et al., 1995; tobacco, Tian and Russell, 1997a; cotton, J. Zhang et al., 1997; *Brassica napus*, Yu et al., 1998; *Plumbago zeylanica*, Tian et al., 2000; *Crocus*, Chichiriccó et al., 2002), the micropyle appears to contain abundant levels of calcium, which correlate closely with fertility and may serve as an attractant in some plants. Accumulations of calcium-induced precipitates of antimonate at the entrance of the micropyle exceeded calcium concentrations in the integument and nucellus in ovules of tobacco and *Plumbago zeylanica*. Calcium distribution appeared to be polarized within the micropylar nucellus, which contained more calcium-induced precipitates than did the chalazal nucellus and thus contribute to an apparent calcium distribution gradient (Tian and Russell, 1997a; Tian et al., 2000).

Some spatial and temporal characteristics are evident in the distribution of loosely bound calcium in the ovule. Calcium concentration in the micropylar regions of each ovule are highest as the pollen tube approaches the ovules in tobacco and *Plumbago zeylanica*. After the tip of the pollen tube passes, calcium levels quickly decline. Flowers in which pollination is withheld, however, retain higher concentrations of micropylar calcium, with calcium levels decreasing slowly over time. That the gradient is retained suggests that micropylar calcium gradients remain. Delayed pollination tests also demonstrate that the ovule continues to attract pollen tubes (Tian and Russell, 1997a; Tian et al., 2000). Accumulating calcium concentrations may be a valuable indicator of fertility (Chudzick and Sniezko, 2003).

Generally, only one pollen tube enters each ovule in angiosperms. In ovaries with only a single ovule, subsequent pollen tubes stop growing once one pollen tube grows into the ovule. However, once multiovulated ovaries are penetrated by pollen tubes, arriving tubes simply bypass the penetrated ovules to grow toward receptive ovules. After the pollen tube

penetrates the ovule, rapid decreases in micropylar calcium correlate closely with loss of receptivity and may prevent supernumerary pollen tubes from entering the ovule. Presumably, the disappearance of micropylar calcium renders penetrated ovules unattractive to the remaining pollen tubes. This latent calcium signal, controlled by penetration of the ovule, can be rapidly dissipated.

#### CALCIUM DISTRIBUTION IN THE EMBRYO SAC

The embryo sac represents the end of the journey for the pollen tube. Because in most angiosperms the pollen tube enters the receptive synergid, calcium distribution in the synergids has been the subject of multiple studies conducted using a variety of calcium detection techniques. The earliest examination of the chemical content of synergids was a study by Jensen (1965) in which sections of the embryo sac of cotton were microincinerated and then examined using dark field microscopy. Abundant ash, presumed to be essentially elemental calcium based on the temperature used, was found localized within the synergids. Since this early work, other techniques have been used to document the abundance of calcium in the synergid of other species. When total calcium in the embryo sacs of wheat and pearl millet before and after pollination was assayed using energy-dispersive X-ray analysis, concentrations greatly exceeded typical physiological measurements; critical-point dried synergid vacuoles contained over 20% calcium by mass (Chaubal and Reger, 1990, 1992a). CTC labeling of membrane-bound calcium in embryo sac cells of tobacco and *Petunia hybrida* indicated intense fluorescent signal in the receptive synergid (Huang and Russell, 1992; Tirlapur et al., 1993). Loosely bound calcium is similarly abundant, according to localizations using potassium antimonate (Chaubal and Reger, 1992a, 1993, 1994; He and Yang, 1992; Tian and Russell, 1997a; Yu et al., 1998; Kristóf et al., 1999). With potassium antimonate, changes in calcium labeling in the receptive synergid can be detected before degeneration and before other ultrastructural cues (Tian and Russell, 1997a). Calcium-induced antimonate precipitates also provide an opportunity to map loosely-bound calcium based on precipitate distribution, size, and quantity (Tian et al., 2000; Yu et al., 1998).

In all flowering plants examined in recent years, except *Plumbago zeylanica* (which lacks synergids), the highest calcium levels within the embryo sac occur in synergids—and typically, the most abundant label is localized in the receptive synergid. Calcium in the synergid appears to be distributed in a micropylar–chalazal gradient. The highest concentration of calcium appears to occur in the filiform apparatus in wheat, pearl millet, tobacco, and *Brassica napus* (Chaubal and Reger, 1992a, b, 1994; Tian and Russell, 1997a; Yu et al., 1998). In sunflower, however, higher concentrations of calcium appear to occur at the chalazal rather than the micropylar end of the synergid (He and Yang, 1992). In all of these plants, the embryo sac contains two synergids, only one of which receives the pollen tube and sperm cells. In mature embryo sacs of wheat, pearl millet, sunflower, and *B. napus*, both sister synergids contain nearly the same calcium content (Chaubal and Reger, 1990, 1992a, b; He and Yang, 1992; Yu et al., 1998). In tobacco and *Petunia hybrida*, however, the two synergids contain different levels of membrane-bound calcium (Huang and Russell, 1992; Tirlapur et al., 1993) as well as

loosely bound calcium (Tian and Russell, 1997a). The degenerated synergid of tobacco contains higher levels of calcium than the persistent synergid (Huang and Russell, 1992). According to Mogensen and Suthar (1978), synergid differences originate during the first day after pollination. Localization of calcium using potassium pyroantimonate confirms a correlation between calcium differences between the two synergids before and during pollen tube penetration (Tian and Russell, 1997a). The pollen tube penetrates the synergid that has the most label, and the pattern of calcium labeling characteristic of the persistent synergid is retained in the unpenetrated synergid after fertilization (Tian and Russell, 1997a).

Abundance of calcium in the synergid is closely related with pollen tube entrance. Although no differences in calcium content are evident in the two synergids in unpollinated flowers of sunflower, calcium content after pollination increases in the synergid that degenerates and receives the pollen tube (He and Yang, 1992). Similarly, calcium content and distribution are similar in the sister synergids of *B. napus* before pollination, but calcium precipitates in both synergids increase by 2.4 times after pollination, and a difference in size of precipitate granules appears to differentiate the two synergids. The synergid that will accept the pollen tube contains smaller granules than the persistent synergid (Yu et al., 1998). In tobacco, calcium levels in the synergid appear to remain elevated until after a pollen tube enters the receptive synergid, when the high level of calcium in the filiform apparatus appears to decrease (Tian and Russell, 1997a). Following pollination in *Torenia fournieri*, the egg apparatus produces a labyrinthine structure on the micropylar surface that contains high levels of calcium. Once the synergid is penetrated by the pollen tube, however, calcium content decreases in the penetrated synergid (Kristóf et al., 1999), and the ovule no longer appears to attract pollen tubes (Higashiyama et al., 1998). In this context, calcium may be only a small part of a larger process of programmed cell death that occurs in the synergid as part of pollen tube arrival and discharge (Christensen et al., 2002).

A continuing interaction between elongating pollen tubes and receptive synergids is evident in terms of calcium signaling. Calcium in the synergid, especially in the filiform apparatus, directly attracts elongating pollen tubes, whereas elongating pollen tubes may stimulate calcium accumulation in the embryo sac (Tian and Russell, 1997a). Similar to calcium dynamics in the micropyle, penetration by a pollen tube induces rapid calcium depletion in the synergids, which may prevent extra pollen tubes from entering the embryo sac (Chaubal and Reger, 1990, 1992b).

Once the pollen tube penetrates the embryo sac, calcium in the degenerated synergid may play a role in sperm cell transport and fusion. In flowering plants, the two sperm cells and vegetative nucleus form a functional association known as the “male germ unit” (Dumas et al., 1984). This assemblage favors the transportation of the male gametes within the tube and assures their simultaneous delivery, but must be severed upon arrival in the embryo sac. Because the assemblage is presumably maintained by cytoskeletal elements (Palevitz and Tiezzi, 1992), calcium in the synergid may be involved in the breakdown of cytoskeletal elements from the pollen tube and the preparation of the sperm cell surface for fusion.

Among the cells of the embryo sac, the egg cell appears to retain consistently low levels of calcium (Chaubal and Reger, 1992a, b; Tian and Russell, 1997a; Yu et al., 1998; Tian et al.,



2000). However, in *Plumbago zeylanica*, which lacks synergids, the egg cell in the mature embryo sac contains abundant calcium, especially in its filiform apparatus (Tian et al., 2000). This result supports the idea that calcium attracts pollen tubes to the ovule and into the embryo sac in vivo and confirms that the egg cell of *P. zeylanica* has indeed acquired some synergid-like functions (Cass and Karas, 1974; Russell, 1982). After fertilization in rice, calcium-induced antimonate precipitates increase by 4.4-fold in the cytoplasm of the zygote and by 10.5-fold in the nucleoplasm. Calcium levels in two-celled proembryos, however, quickly decline in the cytoplasm, but remain abundant in nucleoli and cell walls. An abundance of calcium was reported in the apoplast of the embryo sac after fertilization and during early embryogenesis (Zhao et al., 2002).

The central cell also contains significant accumulations of calcium, particularly at the micropylar end of the cell (Tian and Russell, 1997a; Tian et al., 2000; Yang et al., 2002). After the second sperm cell fuses with the central cell, labeled calcium also quickly decreases in the newly formed endosperm. There seems to be no direct relationship between calcium in the central cell and chemotropic activity of pollen tubes in vivo. Calcium gradients near the synergids presumably control the attraction of pollen tubes, whereas calcium in the central cell may relate more specifically to the stimulation of gamete fusion and the early development of the endosperm, which precedes that of the embryo. Because the central cell is a major nutritional store for the embryo sac, high levels of calcium may also facilitate the absorption of nutrients into the embryo sac. Abundant calcium has been demonstrated to be necessary for accumulation and transport of amino acids (Rickauer and Tauner, 1986) and for carbohydrate metabolism (Brauer et al., 1990). In late stages of microspore development in rice, the anther locule also contains abundant calcium, which appears to be related to rapid starch accumulation (Tian et al., 1998). Additional experimental evidence will be needed to further our understanding of the role of calcium in the central cell.

Knowledge on the function of the antipodal cells is very limited in most plants. In many, the antipodal cells degenerate prior to embryo sac maturity and have no obvious function in the embryo sac. In these plants, antipodal cells presumably contain low levels of calcium, which distinguishes them from the other cells of the embryo sac (Tian and Russell, 1997a). Although antipodal cells in *Plumbago zeylanica* may contain some calcium granules, most are localized in the single large vacuole, with no apparent gradients formed. After fertilization of the central cell, antipodal cells degenerate as the endosperm begins to develop (Tian et al., 2000).

### CALCIUM AND FUSION OF GAMETES

Calcium is known to induce somatic fusion and appears to contribute to gametic fusion as well. In an in vitro fertilization study in maize, the highest fusion success was obtained using 5 mM  $\text{Ca}^{2+}$  in the fusion medium (Faure et al., 1994). Fusion was highly successful in sperm–egg cell pairs (79.8%), whereas only 1.8% of sperm–mesophyll protoplast combinations fused. Under these circumstances, gametes adhered and fused within ~1 min. This fusion specificity has been interpreted as indicative of gametic cell recognition (Faure et al., 1994). Because lowering or eliminating calcium from the medium inhibited fusion and raising the calcium concentration

did not improve fusion success, Faure et al. (1994) concluded that 5 mM calcium stimulated but did not coerce fusion. In contrast, fusion may be coerced at high calcium concentrations (50 mM), particularly when accompanied by high pH (Kranz and Lörz, 1994). Under these conditions, fusions among a variety of cell types are possible, apparently overriding any existing cell recognition systems. Although fusions of isolated tobacco sperm and egg cells could be accomplished using polyethylene glycol without calcium, the addition of 5 mM  $\text{CaCl}_2$  improved success in preliminary in vitro fertilization experiments (Tian and Russell, 1997c). Lower levels of calcium are typically used accompanying electrofusion (e.g., 2 mM  $\text{CaCl}_2$  in rice; Uchiumi et al., 2006).

Calcium alone, however, cannot replace the need to have gametes at the appropriate position in the cell cycle to initiate successful embryogenesis; this cell phase at which fusion normally occurs differs among angiosperms (Friedman, 1999). Although tobacco cells readily form callus and plants are easily regenerated from callus, tobacco gametes isolated at  $G_1$  phase did not successfully form embryos (Tian and Russell, 1997c). A detailed examination of the role of the cell cycle in tobacco fertilization showed that sperm cells underwent S phase upon arrival at the ovule and completed S phase within the synergid. Gametic fusion then occurred at the beginning of the  $G_2$  phase, and completion of S phase in the female gametes was synchronized with the male gametes (Tian et al., 2005). In tobacco, only gametes in which the sperm and egg cells fused at  $G_2$  phase formed successful embryos, whereas male gametes of tobacco isolated from the pollen tube at  $G_1$  phase formed fusion products that failed to divide. Cell cycle congruity is thus a prerequisite for successful in vitro fertilization, even if calcium requirements are met (Wang et al., 2006).

Ultrastructural studies of embryo sacs have frequently revealed highly electron dense materials between the egg and central cells near the site of future gamete fusion. Although it is unclear whether these materials are directly involved in fusion in vivo (Russell, 1992), with the antimonite technique, abundant calcium precipitated at the fusion site in tobacco (Tian and Russell, 1997a), *Brassica napus* (Yu et al., 1998), and *Plumbago zeylanica* (Tian et al., 2000). Presumably, an environment containing sufficient calcium favors the fusion of male and female gametes.

Spontaneous fusion of sperm cell pairs—a condition thought to be rare in nature—may be stimulated in sperm cells in vitro. Using solutions containing 4.27 mM calcium, Roedel and Dumas (1993) found spontaneous sperm cell fusions that formed large, initially multinucleated cells in isolated maize gametes. Under certain culture conditions, media containing as low as 1 mM calcium were able to induce sperm cell fusions consisting of as many as 20 nuclei (G. Zhang et al., 1997). Without calcium in the medium, however, spontaneous fusion of tobacco sperm cells infrequently occurred and took >30 min to complete. In fusion media containing calcium, the frequency was markedly increased, with cells fusing in as little as 10 s, suggesting a direct stimulation of sperm cell fusion (Tian and Russell, 1998). The spontaneous ability of sperm cells to fuse among themselves decreases as the cells mature, suggesting maturational changes in the sperm cell surface during development. Within 20 h of sperm cell formation, spontaneous sperm–sperm fusion is no more frequent than at the time of gamete fusion and requires enzymatic treatment (Tian and Russell, 1998).

Because calcium-mediated prefertilization events are possi-

ble in angiosperm gametes, Williams et al. (1997a, b) examined the uptake kinetics of  $\text{Ca}^{2+}$  and the effect of exogenous calcium on protein phosphorylation and the  $\text{Ca}^{2+}$ -binding proteins, calmodulin and calreticulin, in sperm cells of maize (*Zea mays*) in the presence and absence of  $\text{Ca}^{2+}$ . Calcium uptake kinetics indicated that  $\text{Ca}^{2+}$  influx in maize sperms is mediated by a combination of active (oxidative phosphorylation-dependent)  $\text{Ca}^{2+}$  transporters and  $\text{Ca}^{2+}$  channels (Williams et al., 1997a). With the addition of 1 mM  $\text{Ca}^{2+}$ , the phosphorylation of a 18-kDa protein increased 13-fold after 12 h incubation, calmodulin content increased by 136% in as little as 1 h, and calreticulin increased by 34% after 3 h, as compared to the controls (Williams et al., 1997b).

Calcium conditions during *in vivo* fertilization have not been directly examined in the intact egg cell because of its relative inaccessibility. A calcium requirement, however, seems likely. Because sperm cells usually enter the embryo by means of the synergid, the calcium conditions of this region may represent the normal environment for gamete fusion. If this is the case, the amount of calcium in the immediate environment of the synergid may be remarkably high (Higashiyama, 2002), but the concentration of calcium actually participating in fusion is unknown.

#### ACTIVATION OF THE EGG CELL AND CALCIUM SIGNALING

In animals, the entry of the sperm into the egg cell triggers embryogenesis, but eggs can also be activated by other disturbances in the surface of the egg. The most conspicuous initial step in egg activation is a significant increase in the level of cytosolic free calcium and dramatic oscillations in calcium influx within the fertilized egg cell (Ridgway et al., 1977; Swann and Ozil, 1994; Tang et al., 2000; Sanders et al., 2002). In the brown alga *Fucus serratus*, fertilization of the egg cell is accompanied by uptake of calcium from the external medium, which appears to be necessary for egg cell activation (Roberts and Brownlee, 1995). Calcium uptake by itself can also trigger egg activation, because the addition of the calcium ionophore A23187 to the culture medium will cause nearly half of treated mouse oocytes to undergo parthenogenesis (Nakasaka et al., 2000).

In flowering plants, changes in membrane-bound  $\text{Ca}^{2+}$  in maize egg cells are detectable using chlorotetracycline. Enhanced labeling occurs at the egg plasma membrane and around the egg nucleus prior to gamete fusion (Tirlapur et al., 1995). Following *in vitro* fertilization of a single egg, membrane-bound  $\text{Ca}^{2+}$  labeling with chlorotetracycline increases in the cytoplasm and around the nucleus, but the  $\text{Ca}^{2+}$  signal at the plasma membrane decreases until it is not detectable (Digonnet et al., 1997).

Dramatic changes in free calcium also occur during *in vitro* fertilization of flowering plant gametes. *In vitro* fertilized egg cells of maize displayed significant changes in free  $\text{Ca}^{2+}$  (Digonnet et al., 1997), which are apparently triggered by fusion (Fig. 3). Cytosolic free  $\text{Ca}^{2+}$  was unchanged during gamete positioning and adhesion. Once fusion occurred, however, free  $\text{Ca}^{2+}$  increased for several minutes and then decreased. This transient calcium elevation apparently triggers an influx in extracellular free  $\text{Ca}^{2+}$ , which is non-invasively detectable using an extracellular  $\text{Ca}^{2+}$ -selective vibrating probe (Antoine et al., 2000). On the egg cell surface prior to fusion,

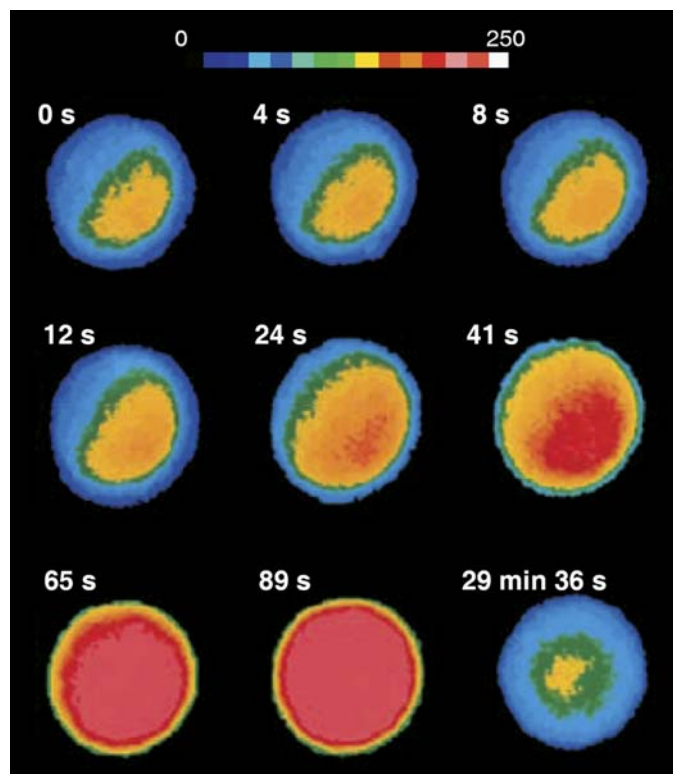


Fig. 3. Pseudocolor ratio imaging of intracellular  $\text{Ca}^{2+}$  concentration of *Zea mays* egg cell loaded with fluo-3 acetoxymethyl ester after *in vitro* fertilization (concentrations in arbitrary fluorescence units). Numbers indicate seconds after sperm-egg fusion, illustrating temporal and spatial distribution of the  $\text{Ca}^{2+}$  transient. Reproduced from Digonnet et al. (1997) with permission of C. Dumas (École Normale Supérieure de Lyon, Lyon, France).

the influx of  $\text{Ca}^{2+}$  was close to zero. Following gamete fusion, however, a  $\text{Ca}^{2+}$  influx was evident near the site of sperm cell entry. The onset of  $\text{Ca}^{2+}$  influx appears to precede the elevation of cytoplasmic  $\text{Ca}^{2+}$  by approximately 40–120 s. The addition of 10  $\mu\text{M}$   $\text{Gd}^{3+}$  prior to fusion is sufficient to block sperm incorporation, suggesting that the  $\text{Ca}^{2+}$  influx is required for incorporation. The fusion point, however, displays a highly localized sperm-dependent and  $\text{Gd}^{3+}$ -insensitive  $\text{Ca}^{2+}$  influx (Antoine et al., 2001).

The role of flowering plant sperm cells in stimulating egg activation may involve chemicals shared with animal egg cells. Using a soluble protein factor of *Brassica* sperm cells, Li et al. (2001) were able to trigger calcium oscillations in mouse eggs and therefore suggested that the activating protein may be found in both plants and animals. Injection of a sperm extract in *Torenia* central cells triggers a calcium oscillation and an accumulation of calcium that mimics endosperm activation. Interestingly, this effect can also be produced by the addition of caged inositol 1,4,5-trisphosphate. Once the cages are photolysed and the chemical is released, inositol 1,4,5-trisphosphate appears to mimic the effects of free calcium oscillation (Han et al., 2002). Stimulation of the female target cells by sperm extract suggests that sperm cells contain a trigger for releasing egg stores of  $\text{Ca}^{2+}$  that may activate a myriad of  $\text{Ca}^{2+}$  channels in both plants and animals. Inositol 1,4,5-trisphosphate is known in other systems to stimulate the

release of  $\text{Ca}^{2+}$  from cellular stores (Depass et al., 2001), and the observation that inositol 1,4,5-trisphosphate can substitute for sperm extract suggests that  $\text{Ca}^{2+}$  accumulation and oscillation may have its origin in signal-transduction signals transmitted by the male during gamete fusion.

In plants as well as animals, there is evidence that fertilization induces the first embryonic events by opening calcium channels and triggering  $\text{Ca}^{2+}$  influx and then calcium oscillation and accumulation, which in turn may trigger further aspects of egg activation (Antoine et al., 2001). These results indicate that spatial and temporal changes in free calcium concentration in fertilized egg cells may be the initial steps in egg cell activation during fertilization in higher plants (Lord and Russell, 2002; Weterings and Russell, 2004).

### CONCLUSION AND FUTURE PROSPECTS

Calcium fulfills many essential roles in sexual reproduction of flowering plants. Interactions between male and female gametes and gametophytes involve different calcium forms, occur at different scales of organization, and require different forms of calcium regulation at each phase. Changing spatial and temporal characteristics of calcium distribution in reproductive tissue and cells reflect the critical role that calcium plays during flowering plant reproduction (Digonnet et al., 1997).

Calcium distributions in pollen tubes closely relate to pollen tube function, polarity, and tip elongation. External stimulation that is locally applied appears to induce asymmetric  $\text{Ca}^{2+}$  distribution in the tube tip and triggers reorientation of the axis of elongation. Subcellular distribution of calcium in the pollen tube occurs along a steep dynamic gradient that is related to tube elongation and cell wall synthesis. When pollen tubes are grown in media containing unsuitable concentrations of calcium or when chemicals are added that block or open calcium channels, the gradient disappears and tube elongation immediately ceases. Although pollen contains some stores of calcium, the much larger pistil of flowering plants contains more abundant calcium stores that support tube elongation requirements in vivo and attract pollen tubes to the ovules and embryo sacs. The spatial and temporal characteristics of calcium distribution and of post-fertilization calcium depletion suggest that calcium accumulation in the ovule provides directional cues that may be impaired if pollination does not occur. This specific form of calcium depletion appears to effectively inhibit fertilization by multiple pollen tubes and may place supernumerary pollen tubes into developmental stasis when all ovules are fertilized. High levels of calcium in degenerated synergids may also function to attract pollen tubes, but further direct experimental evidence is needed. Taken together, these results indicate that the calcium ion plays a critical role during plant reproduction.

Significant progress has been made in understanding the role of calcium in sexual plant reproduction, but there are numerous remaining problems. First, many of the results reported were obtained in vitro. In vitro observations are presumed to reflect the natural state of pollen tubes and to represent gamete behavior, but significant and perhaps critical functional differences may be overlooked. Pollen tubes elongate faster in vivo, tubes live longer, and sperm cells (in bicellular species) form sooner and more reliably than they do in vitro. Confirmatory evidence would either reassure or extend our

current knowledge. Confirmatory results using techniques other than antimonate precipitation should be used to localize loosely bound calcium in pistillate tissues. Even though calcium signaling may be involved in significant short- or long-distance communication, the role of free calcium in pistils is extremely difficult to study directly.

The role of free calcium during embryo sac function is essentially unknown, despite its potential significance during in vivo calcium signaling that may occur between male and female cells during pollen tube growth. In vivo studies of free calcium in ovulo should be particularly useful for expanding our understanding of the role of calcium during fertilization. Particularly attractive as study subjects are the handful of flowering plants that have naturally emergent embryo sacs in which the synergids and egg extend beyond the micropyle (viz., *Torenia fournieri*, *T. hirsuta*, *Philadelphus coronarius*, *Galium lucidum*). These plants provide ideal and intact living materials for examining calcium signals in male and female cells in vivo. The use of in vivo male and female cells to study the behavior and effects of calcium should contribute significantly to our understanding of fertilization mechanisms in angiosperms.

### LITERATURE CITED

- ALMERS, W. 1990. Exocytosis. *Annual Review of Physiology* 52: 607–624.
- ANIL, V. S., AND K. S. RAO. 2001. Calcium-mediated signal transduction in plants: a perspective on the role of  $\text{Ca}^{2+}$  and CDPKs during early plant development. *Journal of Plant Physiology* 158: 1237–1256.
- ANTOINE, A. F., J. E. FAURE, S. CORDEIRO, C. DUMAS, M. ROUGIER, AND J. A. FEIJÓ. 2000. A calcium influx is triggered and propagates in the zygote as a wavefront during in vitro fertilization of flowering plants. *Proceedings of the National Academy of Sciences, USA* 97: 10643–10648.
- ANTOINE, A. F., J. E. FAURE, C. DUMAS, AND J. A. FEIJÓ. 2001. Differential contribution of cytoplasmic  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  influx to gamete fusion and egg activation in maize. *Nature Cell Biology* 3: 1120–1123.
- BATTEY, N. H., AND H. D. BLACKBOURN. 1993. The control of exocytosis in plant cells. *New Phytologist* 125: 307–338.
- BATTEY, N. H., N. C. JAMES, A. J. GREENLAND, AND C. BROWNLEE. 1999. Exocytosis and endocytosis. *Plant Cell* 11: 643–659.
- BAXTER-BURRELL, A., Z. B. YANG, P. S. SPRINGER, AND J. BAILEY-SERRES. 2002. RopGAP4-dependent Rop GTPase rheostat control of *Arabidopsis* oxygen deprivation tolerance. *Science* 296: 2026–2028.
- BEDNARSKA, E. 1989a. The effect of intercellular calcium level regulators on the synthesis of pollen tube callose in *Oenothera biennis* L. *Acta Societatis Botanicorum Poloniae* 58: 39–50.
- BEDNARSKA, E. 1989b. The effects of exogenous  $\text{Ca}^{2+}$  ions on pollen grain germination and pollen tube growth: investigations with  $^{45}\text{Ca}^{2+}$  together with verapamil,  $\text{La}^{3+}$  and ruthenium red. *Sexual Plant Reproduction* 2: 53–58.
- BEDNARSKA, E. 1989c. Localization of calcium on the stigma surface of *Ruscus aculeatus* L.—studies by the chlorotetracycline and X-ray microanalysis. *Planta* 179: 11–16.
- BEDNARSKA, E. 1991. Calcium uptake from the stigma by germinating pollen in *Primula officinalis* L. and *Ruscus aculeatus* L. *Sexual Plant Reproduction* 4: 36–38.
- BERRIDGE, M. J. 1993. Inositol trisphosphate and calcium signaling. *Nature* 361: 315–325.
- BRAUER, M., D. SANDERS, AND M. STITT. 1990. Regulation of photosynthetic sucrose synthesis: a role for calcium? *Planta* 182: 236–243.
- BREWBAKER, J. L., AND B. H. KWACK. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. *American Journal of Botany* 50: 859–865.
- BRINLEY, F. J. JR., T. TIFFERT, A. SCARPA, AND L. J. MULLINS. 1977. Intracellular calcium buffering capacity in isolated squid axons. *Journal of General Physiology* 70: 355–384.

- BUSH, D. S. 1993. Regulation of cytosolic calcium in plants. *Plant Physiology* 103: 7–13.
- BUSH, D. S. 1995. Calcium regulation in plant cells and its role in signaling. *Annual Review of Plant Physiology and Plant Molecular Biology* 46: 95–122.
- CAMACHO, L., AND R. MALHÓ. 2003. Endo/exocytosis in the pollen tube apex is differentially regulated by  $\text{Ca}^{2+}$  and GTPases. *Journal of Experimental Botany* 54: 83–92.
- CASS, D. D., AND I. KARAS. 1974. Ultrastructural organization of the egg of *Plumbago zeylanica*. *Protoplasma* 81: 49–62.
- CHANDLER, J. A., AND S. BATTERSBY. 1976. X-ray microanalysis of zinc and calcium in ultrathin sections of human sperm cells using the pyroantimonate technique. *Journal of Histochemistry and Cytochemistry* 24: 740–748.
- CHAUBAL, R., AND B. J. REGER. 1990. Relatively high calcium is localized in synergid cells of wheat ovaries. *Sexual Plant Reproduction* 3: 98–102.
- CHAUBAL, R., AND B. J. REGER. 1992a. Calcium in the synergid cells and other regions of pearl millet ovaries. *Sexual Plant Reproduction* 5: 34–46.
- CHAUBAL, R., AND B. J. REGER. 1992b. The dynamics of calcium distribution in the synergid cells of wheat after pollination. *Sexual Plant Reproduction* 5: 206–213.
- CHAUBAL, R., AND B. J. REGER. 1993. Prepollination degeneration in mature synergids of pearl millet: an examination using antimonate fixation to localize calcium. *Sexual Plant Reproduction* 6: 225–238.
- CHAUBAL, R., AND B. J. REGER. 1994. Dynamics of antimonate-precipitated calcium and degeneration in unpollinated pearl millet synergids after maturity. *Sexual Plant Reproduction* 7: 122–134.
- CHEUNG, A. Y., C. Y. H. CHEN, L. Z. TAO, T. ANDREYEVA, D. TWELL, AND H. M. WU. 2003. Regulation of pollen tube growth by Rac-like GTPases. *Journal of Experimental Botany* 54: 73–81.
- CHICHIRICÓ, G., A. M. RAGNELLI, AND P. AIMOLA. 2002. Ovary–ovule transmitting tract in *Crocus* (Iridaceae), structure and calcium distribution. *Plant Systematics and Evolution* 235: 155–167.
- CHRISTENSEN, C. A., S. W. GORSICH, R. H. BROWN, L. G. JONES, J. BROWN, J. M. SHAW, AND G. N. DREWS. 2002. Mitochondrial GFA2 is required for synergid cell death in *Arabidopsis*. *Plant Cell* 14: 2215–2232.
- CHUDZIK, B., AND R. SNIEMKO. 2003. Calcium ion presence as a trait of receptivity in tenuinucellar ovules of *Galanthus nivalis* L. *Acta Biologica Cracoviensia Series Botanica* 45: 133–141.
- CRESTI, M., AND A. TIEZZI. 1990. Germination and pollen tube formation. In S. Blackmore and R. B. Knox [eds.], *Microspores: evolution and ontogeny*, 239–263. Academic Press, London, UK.
- CRESTI, M., AND A. TIEZZI. 1992. Pollen tube emission, organization and tip growth. In M. Cresti and A. Tiezzi [eds.], *Sexual plant reproduction*, 89–98. Springer-Verlag, Berlin, Germany.
- DAY, L. A., T. C. WIRT, AND R. D. MORTENSEN. 1971. Atomic absorption spectrophotometric analysis of the calcium ion gradient in *Gladiolus*. *Botanical Gazette* 132: 62–65.
- DEPASS, A. L., R. C. CRAIN, AND P. K. HEPLER. 2001. Inositol 1,4,5 trisphosphate is inactivated by a 5-phosphatase in stamen hair cells of *Tradescantia*. *Planta* 213: 518–524.
- DIGONNET, C., D. ALDON, N. LEDUC, C. DUMAS, AND M. ROUGIER. 1997. First evidence of a calcium transient in flowering plants at fertilization. *Development* 124: 2867–2874.
- DUMAS, C., R. B. KNOX, C. A. MCCONCHIE, AND S. D. RUSSELL. 1984. Emerging physiological concepts in fertilization. *What's New in Plant Physiology* 15: 17–20.
- ELLEMAN, C. J., AND H. G. DICKINSON. 1999. Commonalities between pollen/stigma and host/pathogen interaction: calcium accumulation during stigmatic penetration by *Brassica oleracea* pollen tubes. *Sexual Plant Reproduction* 12: 194–202.
- EVANS, N. H., M. R. MCAINSH, AND A. M. HETHERINGTON. 2001. Calcium oscillations in higher plants. *Current Opinion in Plant Biology* 4: 415–420.
- FAN, L. M., Y. F. WANG, H. WANG, AND W. H. WU. 2001. In vitro *Arabidopsis* pollen germination and characterization of the inward potassium currents in *Arabidopsis* pollen grain protoplasts. *Journal of Experimental Botany* 52: 1603–1614.
- FAURE, J. E., C. DIGONNET, AND C. DUMAS. 1994. An in vitro system for adhesion and fusion of maize gametes. *Science* 263: 1598–1600.
- FEIJÓ, J. A., R. MALHÓ, AND G. OBERMEYER. 1995. Ion dynamics and its possible role during in vitro pollen germination and tube growth. *Protoplasma* 187: 155–167.
- FEIJÓ, J. A., J. SAINHAS, T. HOLDAWAY-CLARKE, M. S. CORDEIRO, J. G. KUNKEL, AND P. K. HEPLER. 2001. Cellular oscillations and the regulation of growth: the pollen tube paradigm. *BioEssays* 23: 86–94.
- FRANKLIN-TONG, V. E. 1999a. Signaling and the modulation of pollen tube growth. *Plant Cell* 11: 727–738.
- FRANKLIN-TONG, V. E. 1999b. Signaling in pollination. *Current Opinion in Plant Biology* 2: 490–495.
- FRANKLIN-TONG, V. E., B. K. DRØBAK, A. C. ALLAN, P. A. C. WATKINS, AND A. L. TREWAVAS. 1996. Growth of pollen tubes of *Papaver rhoeas* is regulated by a slow-moving calcium wave propagated by inositol 1,4,5-trisphosphate. *Plant Cell* 8: 1305–1321.
- FRANKLIN-TONG, V. E., G. HACKETT, AND P. K. HEPLER. 1997. Ratio-imaging of  $\text{Ca}^{2+}$  in the self-incompatibility response of *Papaver rhoeas*. *Plant Journal* 12: 1375–1386.
- FRANKLIN-TONG, V. E., T. L. HOLDAWAY-CLARKE, K. R. STRAATMAN, J. G. KUNKE, AND P. K. HEPLER. 2002. Involvement of extracellular calcium influx in the self-incompatibility response of *Papaver rhoeas*. *Plant Journal* 29: 333–345.
- FRANKLIN-TONG, V. E., J. P. RIDE, AND F. C. H. FRANKLIN. 1995. Recombinant stigmatic self-incompatibility (S-) protein elicits a  $\text{Ca}^{2+}$  transient in pollen of *Papaver rhoeas*. *Plant Journal* 8: 299–307.
- FRANKLIN-TONG, V. E., J. P. RIDE, N. D. READ, A. J. TREWAVAS, AND F. C. H. FRANKLIN. 1993. The self-incompatibility response in *Papaver rhoeas* is mediated by cytosolic free calcium. *Plant Journal* 4: 163–177.
- FRIEDMAN, W. E. 1999. Expression of the cell cycle in sperm of *Arabidopsis*: implications for understanding patterns of gametogenesis and fertilization in plants and other eukaryotes. *Development* 126: 1065–1075.
- FU, Y., G. WU, AND Z. B. YANG. 2001. Rop GTPase-dependent dynamics of tip-localized F-actin controls tip growth in pollen tubes. *Journal of Cell Biology* 152: 1019–1032.
- GEITMANN, A., AND M. CRESTI. 1998.  $\text{Ca}^{2+}$  channel control the rapid expansions in pulsating growth of *Petunia hybrida* pollen tubes. *Journal of Plant Physiology* 152: 439–447.
- GEITMANN, A., J. HUDAK, F. VENNIGERHOLZ, AND B. WALLE. 1995. Immunogold localization of pectin and callose in pollen grains and pollen tubes of *Brugmansia suaveolens*: implications for the self-incompatibility reaction. *Journal of Plant Physiology* 147: 225–235.
- GEITMANN, A., AND E. PARRE. 2004. The local cytomechanical properties of growing pollen tubes correspond to the axial distribution of structural cellular elements. *Sexual Plant Reproduction* 17: 9–16.
- GILROY, S., P. C. BETHKE, AND R. L. JONES. 1993. Calcium homeostasis in plants. *Journal of Cell Science* 106: 453–462.
- GLENK, H. O., W. EAGER, AND O. SCHIMMER. 1971. Can  $\text{Ca}^{2+}$  ions act as a chemotropic factor in *Oenothera* fertilization? In J. Heslop-Harrison [ed.], *Pollen development and physiology*, 255–261. Appleton-Century-Crofts, New York, New York, USA.
- GRADMANN, D. 2001. Models for oscillations in plants. *Australian Journal of Plant Physiology* 28: 577–590.
- GU, Y., V. VERNOUN, Y. FU, AND Z. B. YANG. 2003. ROP GTPase regulation of pollen tube growth through the dynamics of tip-localized F-actin. *Journal of Experimental Botany* 54: 93–101.
- HAN, Y. Z., B. Q. HUANG, F. L. GUO, S. Y. ZEE, AND H. K. GU. 2002. Sperm extract and inositol 1,4,5-trisphosphate induce cytosolic calcium rise in the central cell of *Torenia fournieri*. *Sexual Plant Reproduction* 15: 187–193.
- HE, C. P., AND H. Y. YANG. 1992. Ultracytochemical localization of calcium in the embryo sac of sunflower. *Chinese Journal of Botany* 4: 99–106.

- HEPLER, P. K. 1997. Tip growth in pollen tubes: calcium leads the way. *Trends in Plant Science* 2: 79–80.
- HEPLER, P. K., D. D. MILLER, E. S. PIERSON, AND D. A. CALLAHAM. 1994. Calcium and pollen tube growth. *Current Topics in Plant Physiology* 12: 111–123.
- HESLOP-HARRISON, J. 1987. Pollen germination and pollen tube growth. *International Review of Cytology* 107: 1–78.
- HESLOP-HARRISON, J., AND Y. HESLOP-HARRISON. 1990. Dynamic aspects of apical zonation in the angiosperm pollen tube. *Sexual Plant Reproduction* 3: 187–194.
- HESLOP-HARRISON, J., Y. HESLOP-HARRISON, M. CRESTI, AND F. CIAMPOLINI. 1991. Ultrastructural features of pollen tubes of *Endymion non-scriptus* modified by cytochalasin D. *Sexual Plant Reproduction* 4: 73–80.
- HIGASHIYAMA, T. 2002. The synergid cell: attractor and acceptor of the pollen tube for double fertilization. *Journal of Plant Research* 115: 149–160.
- HIGASHIYAMA, T., H. KUROIWA, S. KAWANO, AND T. KUROIWA. 1998. Guidance in vitro of the pollen tube to the naked embryo sac of *Torenia fournieri*. *Plant Cell* 10: 2019–2031.
- HIGASHIYAMA, T., H. KUROIWA, AND T. KUROIWA. 2003. Pollen-tube guidance: beacons from the female gametophyte. *Current Opinion in Plant Biology* 6: 36–41.
- HOLDAWAY-CLARKE, T. L., J. A. FEJÓ, G. R. HACKETT, J. G. KUNKEL, AND P. K. HEPLER. 1997. Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. *Plant Cell* 9: 1999–2010.
- HOLDAWAY-CLARKE, T. L., N. M. WEDDLE, S. KIM, A. ROBL, C. PARRIS, J. G. KUNKEL, AND P. K. HEPLER. 2003. Effect of extracellular calcium, pH and borate on growth oscillations in *Lilium formosanum* pollen tubes. *Journal of Experimental Botany* 54: 65–72.
- HRUBA, P., D. HONYS, AND J. TUPY. 2005. Expression and thermotolerance of calreticulin during pollen development in tobacco. *Sexual Plant Reproduction* 18: 143–148.
- HUANG, B. Q., AND S. D. RUSSELL. 1992. Synergid degeneration in *Nicotiana*: a quantitative, fluorochromatic and chlorotetracycline study. *Sexual Plant Reproduction* 5: 151–155.
- JAFFE, L. A., M. H. WEISENSEEL, AND L. F. JAFFE. 1975. Calcium accumulations within the growing tips of pollen tubes. *Journal of Cell Biology* 67: 488–492.
- JAUH, G. Y., AND E. M. LORD. 1996. Localization of pectins and arabinogalactan-proteins in lily (*Lilium longiflorum* L.) pollen tube and style, and their possible roles in pollination. *Planta* 199: 251–261.
- JENSEN, W. A. 1965. The ultrastructure and histochemistry of the synergid of cotton. *American Journal of Botany* 52: 238–256.
- JOHNSON, M. A., AND D. PREUSS. 2002. Plotting a course: multiple signals guide pollen tubes to their targets. *Developmental Cell* 2: 273–281.
- JONES, M. A., J. J. SHEN, Y. FU, H. LI, Z. B. YANG, AND C. S. GRIERSON. 2002. The *Arabidopsis* Rop2 GTPase is a positive regulator of both root hair initiation and tip growth. *Plant Cell* 14: 763–776.
- KOHNO, T., AND T. SHIMMEN. 1987. Ca<sup>2+</sup>-induced fragmentation of actin filaments in pollen tubes. *Protoplasma* 141: 177–179.
- KOHNO, T., AND T. SHIMMEN. 1988. Mechanism of Ca<sup>2+</sup> inhibition of cytoplasmic streaming in lily pollen tubes. *Journal of Cell Science* 91: 501–509.
- KRANZ, E., AND H. LÖRZ. 1994. In vitro fertilisation of maize by single egg and sperm cell protoplast fusion mediated by high calcium and high pH. *Zygote* 2: 125–128.
- KRISTÓF, Z., O. TÍMIR, AND K. IMRE. 1999. Changes of calcium distribution in ovules of *Torenia fournieri* during pollination and fertilization. *Protoplasma* 208: 149–155.
- KUNZ, C., A. CHANG, J. D. FAURE, A. E. CLARKE, G. M. POLY, AND M. A. ANDERSON. 1996. Phosphorylation of style S-RNases by Ca<sup>2+</sup>-dependent protein kinases from pollen tubes. *Sexual Plant Reproduction* 9: 25–34.
- KWACK, B. H. 1967. Studies on cellular site of calcium action in promoting pollen tube growth. *Physiologia Plantarum* 20: 825–833.
- KWACK, B. H., AND J. L. BREWBAKER. 1961. The essential role of calcium ion in pollen germination and the population effect. *Plant Physiology* 36 (Supplement): xvi.
- LENARTOWSKA, M., K. KARAS, J. MARSHALL, R. NAPIER, AND E. BEDNARSKA. 2002. Immunocytochemical evidence of calreticulin-like protein in pollen tubes and styles of *Petunia hybrida* Hort. *Protoplasma* 219: 23–30.
- LENARTOWSKA, M., M. I. RODRIGUEZ-GARCIA, AND E. BEDNARSKA. 2001. Calmodulin and calmodulin-like protein are involved in pollen–pistil interaction: immunocytochemical studies on *Petunia hybrida* Hort. *Acta Biologica Cracoviensia Series Botanica* 43: 117–123.
- LI, S. T., X. Y. HUANG, AND F. Z. SUN. 2001. Flowering plant sperm contains a cytosolic soluble protein factor which can trigger calcium oscillations in mouse eggs. *Biochemical and Biophysical Research Communications* 287: 56–59.
- LI, Y. Q., F. CHEN, H. F. LINSKENS, AND M. CRESTI. 1994. Distribution of unesterified and esterified pectins in cell walls of pollen tubes of flowering plants. *Sexual Plant Reproduction* 7: 145–152.
- LORD, E. M., AND S. D. RUSSELL. 2002. The mechanisms of pollination and fertilization in plants. *Annual Review of Cell and Developmental Biology* 18: 81–105.
- MALHÓ, R. 1998a. Expanding tip-growth theory. *Trends in Plant Science* 3: 40–42.
- MALHÓ, R. 1998b. Pollen tube guidance: the long and winding road. *Sexual Plant Reproduction* 11: 242–244.
- MALHÓ, R. 1998c. Role of 1,4,5-inositol triphosphate-induced Ca<sup>2+</sup> release in pollen tube orientation. *Sexual Plant Reproduction* 11: 231–235.
- MALHÓ, R., A. MOUTINHO, A. VAN DER LUIT, AND A. J. TREWAVAS. 1998. Spatial characteristics of Ca<sup>2+</sup> signaling: the calcium wave as a basic unit in plant cell calcium signaling. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 353: 1463–1473.
- MALHÓ, R., N. D. READ, M. S. PAIS, AND A. J. TREWAVAS. 1994. Role of cytosolic free calcium in the reorientation of pollen tube growth. *Plant Journal* 5: 331–341.
- MALHÓ, R., N. D. READ, A. J. TREWAVAS, AND M. S. PAIS. 1995. Calcium channel activity during pollen tube growth and reorientation. *Plant Cell* 7: 1173–1184.
- MALHÓ, R., AND A. J. TREWAVAS. 1996. Localized apical increases of cytosolic free calcium control pollen tube orientation. *Plant Cell* 8: 1935–1949.
- MAO, J. Q., Y. Y. CHEN, AND Y. MIAO. 1992. Ca ion localization in the path of pollen tube growth within the gynoceium of *Brassica napus*. *Acta Botanica Sinica* 34: 233–236.
- MARMÉ, D. 1989. The role of calcium and calmodulin in signal transduction. In F. B. Wendy and D. J. Morré [eds.], *Second messengers in plant growth and development*, 57–80. Alan R. Liss, New York, New York, USA.
- MASCARENHAS, J. P. 1975. The biochemistry of angiosperm pollen development. *Botanical Review* 41: 259–341.
- MASCARENHAS, J. P., AND L. MACHLIS. 1962. Chemotropic response of *Antirrhinum majus* pollen to calcium. *Nature* 196: 292–293.
- MCCLURE, B. A., AND V. FRANKLIN-TONG. 2006. Gametophytic self-incompatibility: understanding the cellular mechanisms involved in “self” pollen tube inhibition. *Planta* 224: 233–245.
- MCCLURE, B. A., J. E. GRAY, M. A. ANDERSON, AND A. E. CLARKE. 1990. Self-incompatibility in *Nicotiana alata* involves degradation of pollen rRNA. *Nature* 347: 757–760.
- MCCNEIL, M., A. G. DARVILL, S. C. FRY, AND P. ALBERSHEIM. 1984. Structure and function of primary cell wall of plants. *Annual Review of Biochemistry* 53: 625–663.
- MESSERLI, M., AND K. R. ROBINSON. 1997. Tip localized Ca<sup>2+</sup> pulses are coincident with peak pulsatile growth rates in pollen tubes of *Lilium longiflorum*. *Journal of Cell Science* 110: 1269–1278.
- MESSERLI, M. A., AND K. R. ROBINSON. 2003. Ionic and osmotic disruptions of the lily pollen tube oscillator: testing proposed models. *Planta* 217: 147–157.
- MILLER, D. D., D. A. CALLAHAM, D. J. GROSS, AND P. K. HEPLER. 1992. Free Ca<sup>2+</sup> in growing pollen tubes of *Lilium*. *Journal of Cell Science* 101: 7–12.
- MOGENSEN, H. L., AND H. K. SUTHAR. 1978. Ultrastructure of the egg

- apparatus of *Nicotiana tabacum* (Solanaceae) before and after fertilization. *Botanical Gazette* 140: 168–179.
- MORRÉ, D. J., AND W. J. VANDERWOUDE. 1974. Origin and growth of cell surface components. In E. D. Hay, T. J. King, and J. Papaconstantinou [eds.], *Macromolecules regulating growth and development*, 81–111. Academic Press, New York, New York, USA.
- MOULINE, K., A. A. VERY, F. GAYMARD, J. BOUCHEREZ, G. PILOT, M. DEVIC, D. BOUCHEZ, J. B. THIBAUD, AND H. SENTENAC. 2002. Pollen tube development and competitive ability are impaired by disruption of a Shaker K<sup>+</sup> channel in *Arabidopsis*. *Genes and Development* 16: 339–350.
- MOUTINHO, A., P. J. HUSSEY, A. J. TREWAVAS, AND R. MALHÓ. 2001. cAMP acts as a second messenger in pollen tube growth and reorientation. *Proceedings of the National Academy of Sciences, USA* 98: 10481–10486.
- MOUTINHO, A., A. J. TREWAVAS, AND R. MALHÓ. 1998. Relocation of a Ca<sup>2+</sup>-dependent protein kinase activity during pollen tube reorientation. *Plant Cell* 10: 1499–1509.
- NAGAI, R., Y. ISHIMA, F. KUKITA, AND T. TAKENAKA. 1975. Calcium and magnesium contents of ectoplasm and endoplasm of *Physarum polycephalum* plasmodium. *Protoplasma* 86: 169–174.
- NAKASAKA, H., S. YAMANO, K. HINOKO, K. NAKAGAWA, M. YOSHIZAWA, AND T. AONO. 2000. Effective activation method with A23187 and puromycin to produce haploid parthenogenones from freshly ovulated mouse oocytes. *Zygote* 8: 203–208.
- NOBILING, R., AND H. D. REISS. 1987. Quantitative analysis of calcium gradients and activity in growing pollen tubes of *Lilium longiflorum*. *Protoplasma* 139: 20–24.
- OBERMAYER, G., AND M. H. WEISENSEEL. 1991. Calcium channel blocker and calmodulin antagonists affect the gradient of free calcium ions in lily pollen tubes. *European Journal of Cell Biology* 56: 319–327.
- PALEVITZ, B. A., AND A. TIEZZI. 1992. Organization, composition, and function of the generative cell and sperm cytoskeleton. *International Review of Cytology* 140: 149–186.
- PARTON, R. M., S. FISCHER-PARTON, A. J. TREWAVAS, AND M. K. WATAHIKI. 2003. Pollen tubes exhibit regular periodic membrane trafficking events in the absence of apical extension. *Journal of Cell Science* 116: 2707–2719.
- PICTON, J. M., AND M. W. STEER. 1983. Evidence for the role of Ca<sup>2+</sup> ions in tip extension in pollen tubes. *Protoplasma* 115: 11–17.
- PIERSON, E. S., Y. LI, H. Q. ZHANG, M. T. M. WILLEMSE, H. F. LINSKENS, AND M. CRESTI. 1995. Pulsatory growth of pollen tubes: investigation of a possible relationship with the periodic distribution of cell wall components. *Acta Botanica Neerlandica* 44: 121–128.
- PIERSON, E. S., D. D. MILLER, D. A. CALLAHAM, A. M. SHIPLEY, B. A. RIVERS, M. CRESTI, AND P. K. HEPLER. 1994. Pollen tube growth is coupled to the extracellular calcium ion flux and the intracellular gradient: effect of BAPTA-type buffers and hypertonic media. *Plant Cell* 6: 1815–1828.
- PIERSON, E. S., D. D. MILLER, D. A. CALLAHAM, J. VAN AKEN, G. HACKETT, AND P. K. HEPLER. 1996. Tip-localized calcium entry fluctuates during pollen tube growth. *Developmental Biology* 174: 160–173.
- POLITO, V. S. 1983. Membrane-associated calcium during pollen grain germination: a microfluorometric analysis. *Protoplasma* 117: 226–232.
- PRING, D., H. TANG, C. CHASE, AND M. SIRIPANT. 2006. Microspore gene expression associated with cytoplasmic male sterility and fertility restoration in sorghum. *Sexual Plant Reproduction* 19: 25–35.
- RATHORE, K. S., R. J. CORK, AND K. R. ROBINSON. 1991. A cytoplasmic gradient of Ca<sup>2+</sup> is correlated with the growth of lily pollen tubes. *Developmental Biology* 148: 612–619.
- REISS, H. D., AND W. HERTH. 1978. Visualization of Ca<sup>2+</sup> gradient in growing pollen tubes of *Lilium longiflorum* with chlorotetracycline fluorescence. *Protoplasma* 97: 373–377.
- REISS, H. D., AND W. HERTH. 1979a. Calcium gradients in tip growing plant cells visualised by chlorotetracycline fluorescence. *Planta* 146: 615–621.
- REISS, H. D., AND W. HERTH. 1979b. Calcium ionophore A23187 affects localized wall secretion in the tip region of pollen tubes of *Lilium longiflorum*. *Planta* 145: 225–232.
- REISS, H. D., AND W. HERTH. 1980. Broad-range effects of ionophore X-537A on pollen tubes of *Lilium longiflorum*. *Planta* 147: 295–301.
- REISS, H. D., AND W. HERTH. 1985. Nifedipine-sensitive calcium channels are involved in polar growth of lily pollen tubes. *Journal of Cell Science* 76: 247–254.
- REISS, H. D., W. HERTH, AND E. SCHNEPP. 1983. The tip-to-base calcium gradient in pollen tubes of *Lilium longiflorum* measured by proton-induced X-ray emission (PIXE). *Protoplasma* 115: 153–159.
- REISS, H. D., AND C. A. MCCONCHIE. 1988. Studies of *Najas* pollen tubes. Fine structure and dependence of chlorotetracycline fluorescence on external free ions. *Protoplasma* 142: 25–35.
- RICKAUER, M., AND W. TANNER. 1986. Effects of Ca<sup>2+</sup> on amino acid transport and accumulation in roots of *Phaseolus vulgaris*. *Plant Physiology* 82: 41–46.
- RIDGWAY, E. B., J. GILKEY, AND L. F. JAFFE. 1977. Free calcium increases explosively in activating medaka eggs. *Proceedings of the National Academy of Sciences, USA* 74: 623–627.
- ROBERTS, S., AND C. BROWNLEE. 1995. Calcium influx, fertilisation potential and egg activation in *Fucus serratus*. *Zygote* 3: 191–197.
- ROBINSON, K. R., AND M. A. MESSERLI. 2002. Pulsating ion fluxes and growth at the pollen tube tip. *Science's STKE* 2002: PE51.
- ROECKEL, P., AND C. DUMAS. 1993. Survival at 20°C and cryopreservation of isolated sperm cells from *Zea mays* pollen grains. *Sexual Plant Reproduction* 6: 212–216.
- ROY, S. J., T. L. HOLDAWAY-CLARK, G. R. HACKETT, J. G. KUNKEL, E. M. LORD, AND P. K. HEPLER. 1999. Uncoupling secretion and tip growth in lily pollen tubes: evidence for the role of calcium in exocytosis. *Plant Journal* 19: 379–386.
- RUDD, J. J., AND V. E. FRANKLIN-TONG. 2001. Unraveling response-specificity in Ca<sup>2+</sup> signaling pathways in plant cells. *New Phytologist* 151: 7–33.
- RUDD, J. J., AND V. E. FRANKLIN-TONG. 2003. Signals and targets of the self-incompatibility response in pollen of *Papaver rhoeas*. *Journal of Experimental Botany* 54: 141–148.
- RUSSELL, S. D. 1982. Fertilization in *Plumbago zeylanica*: entry and discharge of pollen tube in the embryo sac. *Canadian Journal of Botany* 60: 2219–2230.
- RUSSELL, S. D. 1992. Double fertilization. *International Review of Cytology* 140: 357–388.
- SANDERS, D., C. BROWNLEE, AND J. F. HARPER. 1999. Communicating with calcium. *Plant Cell* 11: 691–706.
- SANDERS, D., J. PELLOUX, C. BROWNLEE, AND J. F. HARPER. 2002. Calcium at the crossroads of signaling. *Plant Cell* 14: S401–S417.
- SANDERS, P. M., A. Q. BUI, B. H. LE, AND R. B. GOLDBERG. 2005. Differentiation and degeneration of cells that play a major role in tobacco anther dehiscence. *Sexual Plant Reproduction* 17: 219–241.
- SHEORAN, I. S., K. A. SPROUL, D. J. H. OLSON, A. R. S. ROSS, AND V. K. SAWHNEY. 2006. Proteome profile and functional classification of proteins in *Arabidopsis thaliana* (Landsberg erecta) mature pollen. *Sexual Plant Reproduction* 19: 185–196.
- SHIVANNA, K. R., AND B. M. JOHRI. 1985. The angiosperm pollen: structure and function. Wiley Eastern, New Delhi, India.
- SNEDDEN, W. Q., AND H. FROMM. 2001. Calmodulin as a versatile calcium signal transducer in plants. *New Phytologist* 151: 35–66.
- SPANSWICK, R. M., AND E. J. WILLIAMS. 1965. Ca<sup>2+</sup> fluxes and membrane potentials in *Nitella translucens*. *Journal of Experimental Botany* 16: 463–473.
- STEER, M. W., AND J. M. STEER. 1989. Pollen tube tip growth. *New Phytologist* 111: 323–358.
- STRAATMAN, K. R., S. K. DOVE, T. HOLDAWAY-CLARKE, P. K. HEPLER, J. G. KUNKEL, AND V. E. FRANKLIN-TONG. 2001. Calcium signaling in pollen of *Papaver rhoeas* undergoing the self-incompatibility (SI) response. *Sexual Plant Reproduction* 14: 105–110.
- SWANN, K., AND J. P. OZIL. 1994. Dynamics of the calcium signal that triggers mammalian egg activation. *International Review of Cytology* 152: 183–222.
- SWANSON, R., T. CLARK, AND D. PREUSS. 2005. Expression profiling of



- Arabidopsis* stigma tissue identifies stigma-specific genes. *Sexual Plant Reproduction* 18: 163–171.
- TANDLER, C. J., C. M. LIBANATI, AND C. A. SANCJIS. 1970. The intracellular localization of inorganic cations with potassium pyroantimonate. Electron microscope and microprobe analysis. *Journal of Cell Biology* 45: 355–366.
- TANG, T. S., J. B. DONG, X. Y. HUANG, AND F. Z. SUN. 2000.  $Ca^{2+}$  oscillations induced by a cytosolic sperm protein factor are mediated by a maternal machinery that functions only once in mammalian eggs. *Development* 127: 1141–1150.
- TAYLOR, L. P., AND P. K. HEPLER. 1997. Pollen germination and tube growth. *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 461–491.
- TIAN, H. Q., A. KUANG, M. E. MUSGRAVE, AND S. D. RUSSELL. 1998. Calcium distribution in fertile and sterile anthers of a photoperiod-sensitive genic male-sterile rice. *Planta* 204: 183–192.
- TIAN, H. Q., AND S. D. RUSSELL. 1997a. Calcium distribution in fertilized and unfertilized ovules and embryo sacs of *Nicotiana tabacum* L. *Planta* 202: 93–105.
- TIAN, H. Q., AND S. D. RUSSELL. 1997b. Micromanipulation of male and female gametes of *Nicotiana tabacum*: I. Isolation of gametes. *Plant Cell Reports* 16: 555–560.
- TIAN, H. Q., AND S. D. RUSSELL. 1997c. Micromanipulation of male and female gametes of *Nicotiana tabacum*: II. Preliminary attempts for in vitro fertilization and egg cell culture. *Plant Cell Reports* 16: 657–661.
- TIAN, H. Q., AND S. D. RUSSELL. 1998. The fusion of sperm cells and the function of male unit (MGU) of tobacco (*Nicotiana tabacum* L.). *Sexual Plant Reproduction* 11: 171–176.
- TIAN, H. Q., T. YUAN, AND S. D. RUSSELL. 2005. Relationship between double fertilization and the cell cycle in male and female gametes of tobacco. *Sexual Plant Reproduction* 17: 243–252.
- TIAN, H. Q., Z. ZHANG, AND S. D. RUSSELL. 1998. Isolation of the male germ unit: organization and function in tobacco (*Nicotiana tabacum* L.). *Plant Cell Reports* 18: 143–147.
- TIAN, H. Q., H. ZHU, AND S. D. RUSSELL. 2000. Calcium changes in ovules and embryo sacs of *Plumbago zeylanica* L. *Sexual Plant Reproduction* 13: 11–20.
- TIRLAPUR, U., E. KRANZ, AND M. CRESTI. 1995. Characterisation of isolated egg cells, *in vitro* fusion products and zygotes of *Zea mays* L. using the technique of image analysis and confocal laser scanning microscopy. *Zygote* 3: 57–64.
- TIRLAPUR, U. K., AND S. V. SHIGGAON. 1988. Distribution of  $Ca^{2+}$  and calmodulin in the papillae cells of the stigma surface, visualized by chlorotetracycline and fluorescing calmodulin-binding phenothiazines. *Annals of Botany* 4: 49–53.
- TIRLAPUR, U. K., J. L. VAN WENT, AND M. CRESTI. 1993. Visualization of membrane calcium and calmodulin in embryo sacs *in situ* and isolated from *Petunia hybrida* L. and *Nicotiana tabacum* L. *Annals of Botany* 71: 161–167.
- TREWAVAS, A. 1999. Le calcium, ç'est la vie: calcium makes waves. *Plant Physiology* 120: 1–6.
- TREWAVAS, A., AND R. MALHÓ. 1998.  $Ca^{2+}$  signaling in plant cells: the big network! *Current Opinions in Plant Biology* 1: 428–433.
- UCHIUMI, T., S. KOMATSU, T. KOSHIBA, AND T. OKAMOTO. 2006. Isolation of gametes and central cells from *Oryza sativa* L. *Sexual Plant Reproduction* 19: 37–45.
- VAN IREN, F., L. VAN ESSEN-JOOLEN, P. VAN DER DUYN SCHOUTEN, P. BOERS-VAN DER SLUIJS, AND W. C. DE BRUIJN. 1979. Sodium and calcium localization in cells and tissues by precipitation with antimonate: a quantitative study. *Histochemistry* 63: 273–294.
- WANG, Y. Y., A. KUANG, S. D. RUSSELL, AND H. Q. TIAN. 2006. In vitro fertilization as a tool for investigating sexual reproduction of angiosperms. *Sexual Plant Reproduction* 19: 103–115.
- WATAHIKI, M. K., A. J. TREWAVAS, AND R. M. PARTON. 2004. Fluctuations in the pollen tube tip-focused calcium gradient are not reflected in nuclear calcium level: a comparative analysis using recombinant yellowameleon calcium reporter. *Sexual Plant Reproduction* 17: 125–130.
- WEHLING, P., B. HACKAUF, AND G. WRICHE. 1994. Phosphorylation of pollen proteins in relation to self-incompatibility in rye (*Secale cereale* L.). *Sexual Plant Reproduction* 7: 67–75.
- WETERINGS, K., AND S. D. RUSSELL. 2004. Experimental analysis of the fertilization process. *Plant Cell* 16: S107–S118.
- WICK, S. M., AND P. K. HEPLER. 1982. Selective localization of intracellular  $Ca^{2+}$  with potassium antimonate. *Journal of Histochemistry and Cytochemistry* 30: 1190–1204.
- WILLIAMS, C. M., G. C. ZHANG, AND D. D. CASS. 1997a. Characterization of calcium uptake in isolated maize sperm cells. *Journal of Plant Physiology* 150: 560–566.
- WILLIAMS, C. M., G. C. ZHANG, M. MICHALAK, AND D. D. CASS. 1997b. Calcium-induced protein phosphorylation and changes in levels of calmodulin and calreticulin in maize sperm cells. *Sexual Plant Reproduction* 10: 83–88.
- YANG, J., J. ZHAO, S. P. LIANG, AND H. Y. YANG. 2002. Ultracytochemical localization of calcium in rice central cell before and after fertilization. *Acta Biologica Cracoviensia Series Botanica* 44: 223–230.
- YANG, Z. 1998. Signaling tip growth in plants. *Current Opinion in Plant Biology* 1: 525–530.
- YU, F. L., S. P. LIANG, H. Y. YANG, AND Y. WANG. 1998. Ultracytochemical localization of calcium in micropyle and embryo sac of *Brassica napus* before and after pollination. *Acta Botanica Sinica* 40: 591–597.
- ZHANG, G., M. K. CAMPENOT, L. E. MCGANN, AND D. D. CASS. 1992. Improvement of longevity and viability of sperm cells isolated from pollen of *Zea mays* L. *Plant Physiology* 100: 47–53.
- ZHANG, G., AND D. D. CASS. 1997. Calcium signaling in sexual reproduction of flowering plants. *Recent Research Developments in Plant Physiology* 1: 75–83.
- ZHANG, G., D. LIU, AND D. D. CASS. 1997. Calcium-induced sperm fusion in *Zea mays* L. *Sexual Plant Reproduction* 10: 74–82.
- ZHANG, G., C. M. WILLIAM, M. K. CAMPENOT, L. E. MCGANN, A. J. CUTLER, AND D. D. CASS. 1995. Effects of calcium, magnesium, potassium and boron on sperm cells isolated from pollen of *Zea mays* L. *Sexual Plant Reproduction* 8: 113–122.
- ZHANG, J. S., H. Y. YANG, L. ZHU, AND H. TONG. 1995. Ultracytochemical localization of calcium in the stigma, style and micropyle of sunflower. *Acta Botanica Sinica* 37: 691–696.
- ZHANG, J. S., H. Y. YANG, L. ZHU, AND H. TONG. 1997. Ultracytochemical localization of calcium in the pollen tube track of cotton gynoecium. *Acta Botanica Sinica* 39: 121–125.
- ZHAO, H. P., AND H. Y. REN. 2006. Rop1ps promote actin cytoskeleton dynamics and control the tip growth of lily pollen tube. *Sexual Plant Reproduction* 19: 83–91.
- ZHAO, J., F. L. YU, S. P. LIANG, C. ZHOU, AND H. Y. YANG. 2002. Changes of calcium distribution in egg cells, zygotes and two-celled proembryos of rice (*Oryza sativa* L.). *Sexual Plant Reproduction* 14: 331–337.
- ZHAO, J., H. Y. YANG, AND E. M. LORD. 2004. Calcium levels increase in the lily styler transmitting tract after pollination. *Sexual Plant Reproduction* 16: 259–263.
- ZONIA, L., S. CORDEIRO, AND J. A. FEJÓ. 2001. Ion dynamics and hydrodynamics in the regulation of pollen tube growth. *Sexual Plant Reproduction* 14: 111–116.
- ZONIA, L., S. CORDEIRO, J. TUPY, AND J. A. FEJÓ. 2002. Oscillatory chloride efflux at the pollen tube apex has a role in growth and cell volume regulation and is targeted by inositol 3,4,5,6-tetrakisphosphate. *Plant Cell* 14: 2233–2249.
- ZOREC, R., AND M. TESTER. 1992. Cytoplasmic calcium stimulates exocytosis in a plant secretory. *Biophysics Journal* 63: 864–867.