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REVIEW PAPER

Spatial and temporal integration of signalling networks regulating pollen tube growth

Laura Zonia*

University of Amsterdam, Swammerdam Institute for Life Sciences, Section of Plant Physiology, Kruislaan 904, 1098 XH Amsterdam, The Netherlands

* To whom correspondence should be addressed. E-mail: l.e.zonia@uva.nl

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Abstract

The overall function of a cell is determined by its contingent of active signal transduction cascades interacting on multiple levels with metabolic pathways, cytoskeletal organization, and regulation of gene expression. Much work has been devoted to analysis of individual signalling cascades interacting with unique cellular targets. However, little is known about how cells integrate information across hierarchical signalling networks. Recent work on pollen tube growth indicates that several key signalling cascades respond to changes in cell hydrodynamics and apical volume. Combined with known effects on cytoarchitecture and signalling from other cell systems, hydrodynamics has the potential to integrate and synchronize the function of the broader signalling network in pollen tubes. This review will explore recent work on cell hydrodynamics in a variety of systems including pollen, and discuss hydrodynamic regulation of cell signalling and function including exocytosis and endocytosis, actin cytoskeleton reorganization, cell wall deposition and assembly, phospholipid and inositol polyphosphate signalling, ion flux, small G-proteins, fertilization, and self-incompatibility. The combined data support a newly emerging model of pollen tube growth.

Key words: Cell hydrodynamics, cell wall, cytoskeleton, endocytosis, exocytosis, fertilization, GTPases, ion flux, lipid signalling, polyphosphoinositides.

Introduction

At any given moment, the readout of cell function or behaviour is a composite of events occurring at different scales—from the level of quantum noise through atomic, molecular, and structural levels. Cells utilize signal transduction cascades to relay information across different scales and from the cell periphery to the interior. Many cellular behaviours, such as pollen tube growth, are orchestrated by multiple signals interacting in a broader signalling network. Order is achieved by integration of these signalling networks with metabolic pathways, morphological and structural rearrangements, and gene expression profiles. Considerable work has been invested in understanding the regulation of individual signal transduction cascades. However, little is known about how cells integrate, regulate, and decode information contained in multiscale hierarchical signalling networks. Recent work on pollen tube growth has identified an important role for volume sensing and cell hydrodynamics as a central mechanism that regulates several key signalling cascades, and has the potential to integrate and synchronize the function of the broader signalling network (Zonia and Munnik, 2007, 2009).

Cells expend substantial resources in sensing, responding to, and adapting to osmotic perturbations and regulating cell volume and cell hydrodynamics. Volume and osmotic changes affect the mechanical/chemical status of the plasma membrane, cellular ionic strength and/or concentrations of specific ions, macromolecular crowding, and proximity of protein-binding partners (Hoffmann *et al.*, 2009). Cell hydrodynamic regulation is linked through feedback control loops to multiple signal transduction cascades including ion fluxes and channels, phospholipids, lipid kinases and lipases, protein kinases and phosphatases, small GTP-binding proteins, cytoskeletal reorganization, reactive oxygen species, osmolyte transport and synthesis, membrane

trafficking, and transcriptional regulation (Hoffmann *et al.*, 2009). New methodologies are enabling real-time visualization of live cell hydrodynamics, and the results show that different regions of the cytoplasm have different water diffusion rates due to differences in macromolecular crowding and structure (Potma *et al.*, 2001*a*, *b*).

The overall goal of this review is not an in-depth examination of key signalling and structural elements that are known to regulate pollen tube growth. Rather, this review intends to explore recent work, particularly on cell hydrodynamics, vesicle trafficking, cell wall synthesis, cytoskeletal behaviours, ion dynamics, Rho GTPase, fertilization, and self-incompatibility, and propose new mechanisms that potentially integrate the spatial and temporal activity of key signalling and structural elements into a unified functional network that determines pollen tube growth.

Cell hydrodynamics, cell morphology, and growth

The cytoplasm has classically been modelled as a viscoelastic or viscoplastic continuum. In this purely hypothetical approximation, the cell interior is considered to be a single homogeneous phase, and changes in osmotic pressure are propagated equally and instantaneously throughout the entire cytoplasm (Fig. 1A). Clearly, this coarse approximation fails to quantify accurately the cytoplasm of a living cell that has two distinct phases—a fluid phase composed of water, ions, soluble proteins, and metabolites, and a solid phase composed of cytoskeletal polymers, endomembrane networks, vesicles, organelles, and particulate matter (Fig. 1B). This section will highlight recent work in various systems that is now refining our understanding of the physical properties of the cytoplasm and cell hydrodynamics.

Examination of blebbing (localized spherical expansion of the cell surface) in mammalian cells and *Dictyostelium* led to the discovery that cell hydrostatic pressure is non-uniform (Charras *et al.*, 2005; Langridge and Kay, 2006). These studies revealed that hydrostatic pressure can be highly asymmetrical, and a localized increase in hydrostatic pressure can induce rupture of the plasma membrane from the underlying cytoskeleton followed by hydrodynamic flow and inflation of the detached membrane. Based on these results, a new poroelastic model has been proposed for the cytoplasm. The poroelastic model considers the cytoplasm to be similar to a fluid-filled sponge, composed of a porous, contractile, elastic solid penetrated by an electrolytic interstitial fluid that moves through the pores in response to electrochemical or pressure gradients (Charras *et al.*, 2005; Spitzer and Poolman, 2005; Mahadevan, 2008; Mitchison *et al.*, 2008). The non-uniform nature of the contractile elastic solid and the electrolytic interstitial fluid results in vectorial pressure and electrochemical gradients, so that the propagation of osmotic pressure is highly dependent on the hydraulic and electrochemical conductivity, the mesh density and channel diameter, and the elastic contraction of the porous network (Mitchison *et al.*, 2008; Spagnoli *et al.*, 2008). It is important to note that these formulations are based on the incompressibility of both the solid and fluid phases. The time dependency to reach pressure equilibrium is a function of cell size and cytoplasmic diffusion constant, such that it takes longer to reach pressure equilibrium in large cells (such as elongated pollen tubes) and in cytoplasmic regions corresponding to a stiff, dense mesh with narrow pores, infiltrated with very viscous fluid (Mahadevan, 2008; Mitchison *et al.*, 2008). In all cell systems examined to date, asymmetrical pressure gradients are easily achieved—both theoretically and experimentally—on physiologically realistic time and length scales of the order of 10–100 s and 5–10 μm, respectively (Charras *et al.*, 2005; Langridge and Kay, 2006; Mahadevan, 2008; Mitchison *et al.*, 2008; Spagnoli *et al.*, 2008).

Recent compelling evidence in support of the poroelastic fluid-filled sponge model was obtained from an elegant study of the movement of quantum dots (diameter ~28 nm) and

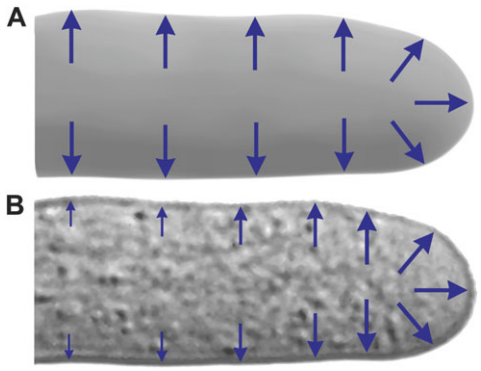

Fig. 1. Theory and modelling of pollen tube cytoplasm and osmotic pressure. (A) A semi-cylindrical viscoelastic continuum. The classic model of pollen tube growth simplified biological complexity by approximating the cytoplasm as composed of a single-phase viscoelastic continuum, in which osmotic pressure propagates equally and instantaneously throughout all parts of the cell. In this coarse approximation, osmotic pressure is uniform throughout all parts of the cell and distributed equally and uniformly along the entire plasma membrane (blue arrows). (B) A living pollen tube. The newly emerging hydrodynamic model of pollen tube growth considers a living cell with a crowded cytoplasm consisting of two distinct phases: a solid phase of vesicles, organelles, endomembrane networks, cytoskeletal polymers, and particulate matter; and a fluid phase composed of water, ions, soluble proteins, and metabolites. Theory and experiment agree that the cytoplasm of living cells conforms to a fluid-filled poroelastic network, in which propagation of osmotic pressure is highly dependent on the local hydraulic and electrical conductivity, the local mesh density and channel size, and the elastic contraction of the porous network. In living cells, theory and experiment have conclusively demonstrated that non-equilibrium osmotic pressure exists on physiologically relevant temporal and spatial scales. In the new hydrodynamic model of pollen tube growth, spatial non-equilibrium osmotic pressure is predicted to be highest near the apex and diminish toward the distal tube (blue arrows).

natural organelles during inhomogeneous osmotic manipulation of HeLa cells (Charras *et al.*, 2009). The cells were subjected to a localized external hypertonic gradient that forced hydrodynamic flow into the cell on one side and out of the cell on the other side. This forced contraction and displacement of cytoplasm on the dehydrated side of the cell while the unexposed side of the cell was essentially unaffected. This result indicates the presence of a boundary between hyperosmotic and iso-osmotic regions of the cytoplasm, with different hydraulic regions co-existing within a few micrometres and on time scales of tens of seconds. Visual tracking of the degree of movement of quantum dots and natural organelles confirmed a gradient of hydration within the cytoplasm. When the entire cell was subjected to hypertonic shock, the movement of both quantum dots and natural organelles was inhibited as the cytoplasmic pore size collapsed due to whole-cell water efflux and the resultant macromolecular crowding. The observed movements during inhomogeneous osmotic manipulation and osmotic shock were the same in both normal and metabolically poisoned cells, demonstrating that the responses are primarily physical rather than metabolic. A further surprising result of this study was that water flows across the membrane faster than through the cytoplasm. In summary, this study strongly supports a poroelastic model of the cytoplasm and spatio-temporal asymmetry in cell hydrodynamics.

Spatial asymmetries in cell hydrodynamics exist in tobacco pollen tubes (Fig. 1B). Hypotonic challenge induces rapid swelling in the apical 50 μm region while the diameter of the distal tube remains unaffected (Zonia *et al.*, 2002, 2006; Zonia and Munnik, 2004, 2007). This effect is not simply due to cell wall properties, as disruption of either pectin, cellulose synthesis, actin, or microtubules induces swelling confined to the apical region in *Solanum*, conifer, lily, and *Papaver* pollen tubes (Anderhag *et al.*, 2000; Geitmann *et al.*, 2000; Vidali *et al.*, 2001; Lazzaro *et al.*, 2003; Parre and Geitmann, 2005). The entire apical region spanning from the tip to 50 μm distal to the tip can undergo essentially immediate volume increases of up to 59% greater than normal before the increased hydrostatic pressure induces rupture of the cell wall and bursting in tobacco pollen tubes (Zonia *et al.*, 2002). Conversely, hypertonic challenge induces a rapid apical volume decrease of up to 41% less than normal volume (Zonia and Munnik, 2004). Together, these results indicate that water flow across the plasma membrane into or out of the cell is rapid, increased pressure can easily drive expansion of the cell wall, and decreased pressure leads to contraction of the cell wall.

Further work quantified temporal asymmetries in cell hydrodynamics in tobacco pollen tubes undergoing oscillatory growth (Fig. 2) (Zonia *et al.*, 2006). Apical volume oscillates with the same frequency as growth rate oscillations, but the

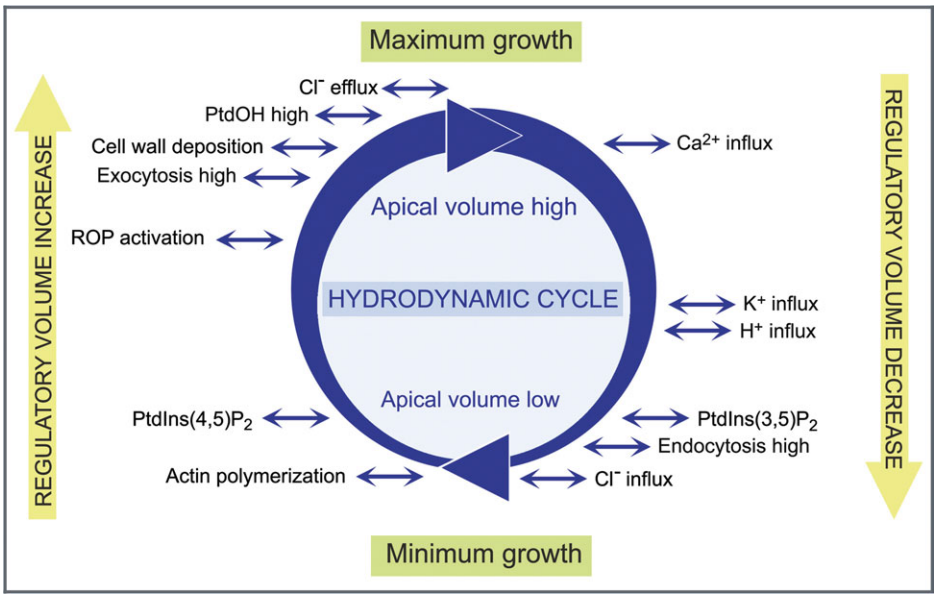

Fig. 2. Integration of signalling and structural networks regulating pollen tube growth. Temporal asymmetries in cell osmotic pressure exist due to metabolism and thermodynamic non-equilibrium, which induce continual fluctuations in osmotic potential across the plasma membrane. This induces hydrodynamic flow into or out of the cell, and affects the mechanical/chemical status of the plasma membrane, cellular ionic strength, concentrations of specific ions, macromolecular crowding, and proximity of protein-binding partners. Signalling cascades and structural elements that are involved in stabilizing cell volume and pressure are activated, in a network of coupled reactions that control regulatory volume decrease in response to cell swelling and regulatory volume increase in response to cell shrinking. Experimental data in tobacco pollen tubes show that hydrodynamics behaves as a limit cycle oscillator that entrains growth rate oscillation frequencies. Thus, the hydrodynamic cycle has the potential to function as a travelling wave that integrates the network of coupled oscillators that are involved in pollen tube growth. Each element in the network could be activated in response to a certain osmotic status and input from other elements in the network. The illustration presents a selection of key signalling cascades and structural elements involved in pollen tube growth.

cycles are phase-shifted by ~180 °. During each growth cycle, apical volume reaches maximum concurrently with the phase of decreasing growth, and reaches minimum concurrently with the phase of maximally increasing growth. Substitution of H_2O with ^2H_2O (deuterium oxide) severely disrupts pollen tube growth and growth rate oscillation frequencies, and induces abnormal pollen tube morphologies (Zonia *et al.*, 2006). These results suggest a mechanism of hydrodynamic loading/unloading during each cycle that is necessary for pollen tube growth. To test this hypothesis, apical volume and growth rate oscillation frequencies were monitored during hypertonic or hypotonic challenge (Zonia *et al.*, 2006). Hypertonic treatment induced growth rate and apical volume oscillation frequencies to halve, corresponding to shifts in growth cycle periods from ~50 s to 100 s. Hypotonic treatment induced growth rate and apical volume oscillation frequencies to double, corresponding to shifts in growth cycle periods from ~50 s to 25 s and 12 s. The observed period doubling and period halving are characteristic properties of limit cycle oscillators that carry frequency-encoded information, and provide strong evidence that hydrodynamic oscillations are closely correlated with or in fact form the basis of the pollen tube oscillator that drives growth rate oscillations and oscillations in all other non-linear circuits linked with growth (e.g. Ca^{2+} flux and Ca^{2+} gradient) (Fig. 2) (Table 1). It is crucial to note that if hydrodynamics was not the oscillator driving growth oscillations (and, for example, cell wall loosening was the true oscillator), then changing hydrodynamic flow would only change the amplitude of each growth cycle, and not the frequency of growth oscillations. Interestingly, recent theoretical analyses suggest that cell hydrodynamics and volume regulation implicitly lead to non-linearity in biochemical and signalling pathways, and forms the basis for emergence of whole-cell oscillatory dynamics (Martin *et al.*, 2009). The result on hypotonic forcing of growth rate oscillation frequencies has recently been re-confirmed in lily pollen tubes (Zerzour *et al.*, 2009). This study also showed that, as expected, manipulation of cell wall properties can lead to changes in growth rate oscillation frequencies. However, there were no data to show dynamically meaningful shifts in growth rate oscillation frequencies as previously shown for hydrodynamic manipulation. Finally, evidence of hydrodynamic efflux at the pollen tube apex was obtained

Table 1. Selective overview of regulation of cell signaling by osmotic pressure and cell volume

Signal/element	High osmotic pressure, cell swelling[a,b]	Low osmotic pressure, cell shrinking[a,c]	Experimental system
Pollen tube growth	Stimulation	Attenuation	Tobacco, lily
Exocytosis	Stimulation	Attenuation	Pollen tubes, guard cells, all vertebrate cells tested
Endocytosis	Attenuation	Stimulation	Pollen tubes, guard cells, all vertebrate cells tested
Secretion and assembly of cell wall pectin	Stimulation	N.D.	Pollen tubes[d], *Chara*
Actin polymerization (net)	Decrease	Increase	Pollen tubes[d], guard cells, non-adherent vertebrate cells
PtdOH	Increase	Decrease	Pollen tubes, unicellular green algae, all vertebrate cells tested
PtdIns	Decrease	No detectable change[e]	Pollen tubes
$PtdIns(4,5)P_2$	Decrease	Increase	Pollen tubes[f], all vertebrate cells tested
$PtdIns(3,5)P_2$	No detectable change[e]	Increase	Pollen tubes, plant cells
$Ins(1,4,5)P_3$	ND	Increase	*Arabidopsis* cell culture, seedlings, leaves; carrot cell culture
$Ins(3,4,5,6)P_4$	Decrease[d]	Increase[d]	Pollen tubes[d], epithelial cells
$InsP_6$	ND	Increase	Pollen tubes[g], guard cells, duckweed turions
Cl^- influx	Attenuation	Stimulation	Plant cells, animal cells, bacteria; all cell types tested
Cl^- efflux	Stimulation	Attenuation	Plant cells, animal cells, bacteria; all cell types tested
Ca^{2+} influx	Stimulation	Attenuation	Plant cells, animal cells, bacteria; all cell types tested
K^+ influx	Attenuation	Stimulation	Plant cells, animal cells, bacteria; all cell types tested
K^+ efflux	Stimulation	Attenuation	Plant cells, animal cells, bacteria; all cell types tested
H^+ influx	Stimulation	Attenuation	Plant cells, animal cells, bacteria; all cell types tested
H^+ efflux	Attenuation	Stimulation	Plant cells, animal cells, bacteria; all cell types tested
Rho family GTPases	Attenuation	Stimulation	Pollen tubes?, epithelial cells
Pollen grain germination on stigma	Stimulation[h]	Inhibition[h]	*Brassica*, *Arabidopsis*
Sperm cell discharge from pollen tube	Stimulation	ND	*Torenia*, *Arabidopsis*

[a] Cell shrinking, swelling can be either global or localized volume changes.
[b] Cell swelling induces in-plane membrane tension, low molecular crowding, low cytosolic ionic strength.
[c] Cell shrinking induces invagination of the plasma membrane, high molecular crowding, high cytosolic ionic strength.
[d] Indirect evidence.
[e] Measurements from whole-cell assays; localized changes in levels or turnover may occur but would be undetectable by the methods used.
[f] In pollen tubes, $PtdIns(4,5)P_2$ increases with hyperosmosis but no detectable change was measured after hypo-osmosis (see note e)
[g] $InsP_6$ levels are high in pollen grains and tubes; responses to changes in pressure have yet to be determined
[h] Self-incompatible pollen grains hydrate and germinate; self pollen grains fail to hydrate and fail to germinate
ND, not determined

with Raman microscopy using microinjected ^2H_2O as a tracer (Zonia *et al.*, 2006).

Evidence of a role for non-equilibrium hydrodynamics in growth of other plant cells and tissues is emerging. An interesting examination of shoot apical meristem architecture in *Arabidopsis* (Corson *et al.*, 2009) supports the prediction of a role for asymmetrical osmotic pressure during plant cell growth and differentiation (Zonia and Munnik, 2007). The study shows that in fact, correct *in planta* growth patterns could only be theoretically modelled and simulated when turgor pressure was not uniform (Corson *et al.*, 2009).

Exocytosis and endocytosis in pollen tube growth

Progress in the understanding of molecular events involved in secretory and enodosomal trafficking in plants has been discussed in recent reviews (Lam *et al.*, 2007; Foresti and Denecke, 2008; Grefen and Blatt, 2008; Nielsen *et al.*, 2008; Moscatelli and Idili, 2009; Richter *et al.*, 2009). Pollen tubes have extremely active vesicle trafficking, with high rates of exocytosis to support rapid growth rates, and endocytosis to internalize excess membrane and recycle proteins (Samaj *et al.*, 2006; Cheung and Wu, 2008). The long-standing model for pollen tube growth considered that exocytosis occurred at the apex, mediated by the pool of very small vesicles in the apical dome that were thought to be secretory vesicles, and endocytosis occurred distally. However, three recent reports support a shift in our understanding of pollen tube growth.

Report I examined the ultrastructural details of uptake and trafficking of positively and negatively charged nanogold in tobacco pollen tubes (Moscatelli *et al.*, 2007). Positively charged nanogold was first observed in distal regions of pollen tubes, ~10 μm from the tip, while negatively charged nanogold was first observed near the apex. During longer incubations, negatively charged nanogold labelled vacuole-like compartments, while positively charged nanogold labelled vesicles close to the *trans*-Gogi complex and the rims of *cis* and medial cisternae, and vesicles containing dense cell wall-like material. These results suggest the presence of two functionally distinct endocytosis pathways. Ikarugamycin, an inhibitor of clathrin-dependent endocytosis, interfered with trafficking to both Golgi and vacuole, but did not block all trafficking to the vacuole, suggesting that a clathrin-independent endocytic pathway also exists in pollen tubes.

Report II employed pulse–chase labelling with two different lipophilic styryl dyes (FM 4-64 and FM 1-43) that have different excitation and emission wavelengths. This strategy enabled visualization of the subcellular localization and dynamics of three distinct populations of vesicles in growing tobacco pollen tubes—two endocytic pathways and the site of exocytosis and the growth zone (Zonia and Munnik, 2008*a*, *b*, 2009). The results revealed that the entire apical dome is a region of endocytosis, which labels within 1–2 min, and the pool of very small vesicles in the apical dome are endocytic vesicles, which label within 3–5 min (Fig. 2). These vesicles form the characteristic inverse-cone pool of vesicles that undergo retrograde trafficking, with the entire population of endosomal trafficking vesicles in the inverse-cone labelling within 5–10 min. A second pathway of endocytosis was observed in distal regions of pollen tubes, starting at ~6 μm distal to the tip. These vesicles are much larger than those at the apex and their rate of internalization is substantially slower. Based on the vesicle sizes and kinetics of internalization, the results indicate that smooth vesicle endocytosis occurs at the apex and clathrin-coated vesicle internalization occurs in the distal region. Exocytosis was observed by dynamic tracking of the insertion of discretely labelled vesicles into the plasma membrane in the subapical region, which is the location of the dense actin fringe (Fig. 3) (for further details on actin arrays see the section 'Cytoskeleton and cell stiffness during swelling and growth'). Exocytosis in the subapical growth zone was also observed using refraction-free high-resolution time-lapse differential interference contrast microscopy, and examination of both individual images and compressed videos (Zonia and Munnik, 2008*a*, *b*). Exocytic vesicles were estimated to have diameters of the order of ~400 μm, in agreement with a previous electron microscopy report that estimated diameters of exocytic vesicles in tobacco pollen tubes at ~400–500 μm (Kroh and Knuiman, 1985). This size renders the vesicles well suited to carry bulky cargoes of membrane proteins and cell wall polymers [e.g. the length of the pectin polygalacturonic acid is estimated at ~100 μm and contains up to 200 GalA residues (Carpita and Gibeaut, 1993)]. The subapical growth zone spans the region from 3 μm to 10 μm distal to the tip, and it is highly dynamic and responsive to changes in growth rate. During periods of rapid growth, many vesicles undergo exocytosis in the mid-zone or trailing edge of the growth zone (~5–10 μm distal to the apex). During periods of slow growth or following transient growth arrest, many vesicles undergo exocytosis near the leading edge of the growth zone (~3–5 μm distal to the apex) (Zonia and Munnik, 2008*a*, 2009). In normally growing pollen tubes, there was no evidence of exocytosis at the apex. Hyperosmotic treatment (see below) revealed information about plasma membrane flow from the site of exocytosis in the subapex—membrane flow is primarily anterograde with uptake/recycling along the apex, and a small degree of retrograde flow. Anterograde membrane flow combined with endocytic recycling along the apex results in the appearance of secreted proteins in the pool of small vesicles in the apical dome (Zonia and Munnik, 2009). Together, these results support a new model of pollen tube growth, with exocytosis in the subapex, smooth vesicle endocytosis along the apical dome, the pool of very small vesicles in the apical dome are endosomal, and clathrin-mediated endocytosis occurring distally.

Report III used FM 1-43 to label vesicles in growing lily pollen tubes, to visualize the speed and direction of movement of labelled vesicles with time-lapse high-speed confocal microscopy and spatiotemporal image correlation

analysis (Bove *et al.*, 2008). Pollen tubes were labelled for 5 min before extensive washout of the fluorophore and transfer of washed pollen tubes to agarose medium for mounting on slides. The vector maps showed overall vesicle motion patterns, with prominent retrograde trafficking of vesicles away from the apex in the inverted cone. Another prominent pool of vesicles showed anterograde trafficking along the cortex to the subapical region, where they appeared to change direction, moving toward the centre and reversing. Vesicles undergoing anterograde trafficking (presumably exocytic vesicles) were observed to accumulate in the subapical region corresponding to the location of the actin fringe. This corresponds to the active growth zone identified in Report II for tobacco pollen tubes. To identify short-range or erratic motion of vesicles in the apical dome, Report III reduced the window size and time scale of the analysis. This showed a diminishingly small degree of anterograde vesicle motion from the subapex into the apical dome. It is important to note that the methods used did not allow visualization of actual exocytosis or endocytosis events. Nevertheless, in their model the authors agree that the apex is the site of smooth vesicle endocytosis and that clathrin-mediated endocytosis occurs distally. They suggest that exocytosis occurs in the shoulder of the apical dome (this corresponds to the leading edge of the growth zone identified in Report II), or a form of fast exocytosis without complete incorporation of the vesicle membrane (termed kiss-and-run) occurs at the apical plasma membrane.

Cell swelling and shrinking has profound affects on plasma membrane architecture and in-plane tension, and is known to stimulate vesicle incorporation (cell swelling) or retrieval (cell shrinking) in many cell types. Hypotonic treatment induces increased growth rates in populations of pollen tubes and increases growth rate oscillation frequencies (Zonia *et al.*, 2002, 2006; Zerzour *et al.*, 2009), while hypertonic treatment decreases growth rates in populations of pollen tubes and decreases growth rate oscillation frequencies (Zonia *et al.*, 2002, 2006) (Table 1). This indicates that growth is tightly linked with hydrodynamics, and if so, then osmotic perturbation should affect vesicle trafficking dynamics. This hypothesis was tested in Report II by examination of vesicle trafficking dynamics in pollen tubes subjected to osmotic manipulation (Zonia and Munnik, 2008*a*). The results showed that hypotonic treatment attenuated smooth vesicle endocytosis at the apex and stimulated exocytosis, supporting the increased growth rate by a higher rate of secretion and reduced uptake/recycling (Table 1). During oscillatory growth, the start of the growth cycle occurs when the apical volume is highest (Zonia *et al.*, 2006). Thus, the combined data indicate a temporal correlation linking exocytosis and the start of the growth cycle (Fig. 2). Conversely, hypertonic treatment greatly stimulated smooth vesicle endocytosis at the apex and inhibited exocytosis, supporting contraction of the protoplast during water efflux (Table 1). During oscillatory growth, the apical volume is lowest during the phase of minimum growth, consistent with the data showing stimulation of endocytosis and attenuation of exocytosis under these conditions (Fig. 2). Clathrin-mediated endocytosis in distal regions was only slightly dampened by hypotonic treatment, but was stimulated by hypertonic treatment. In summary, these results are consistent with a central role for hydrodynamics in the regulation of vesicle trafficking, modulation of the rates of exocytosis and endocytosis, and integration of exocytosis and endocytosis rates with the growth rate (Zonia and Munnik, 2008*a*, 2009).

Despite these recent reports, the subcellular localization of exocytosis remains controversial. A recent study used fluorescence recovery after photobleaching and a secreted green fluorescent protein–receptor-like kinase (GFP–RLK) in tobacco pollen tubes, and reports evidence in support of exocytosis at the apex (Lee *et al.*, 2008*c*). During the first 30 s after photobleaching, the pollen tube grew slightly; there was moderate recovery of fluorescence along the apical dome and minimal recovery of fluorescence in the shoulder of the apical dome. During 30–60 s after photobleaching, the pollen tube stopped growing, there was a large increase in fluorescence along the apical dome, and a corresponding increase in fluorescence in the shoulder of the apical dome (although the levels measured in the shoulder remained lower than along the apical dome). By 90 s after photobleaching, the pollen tube again grew slightly and fluorescence in the shoulder of the apical dome was comparable with fluorescence along the apical dome. These results were interpreted as evidence of exocytosis at the apex, thus supporting the long-standing model of pollen tube growth.

Cell wall deposition and assembly in pollen tube growth

Despite recent progress in our understanding of the regulation and biosynthesis of plant cell walls (Geisler *et al.*, 2008; Szymanski and Cosgrove, 2009), there are still knowledge gaps, particularly regarding the biochemistry of *in muro* wall synthesis. The long-standing model of pollen tube growth considers that growth is ultimately dependent on biochemical modification (called cell wall loosening) of the wall at the apex, where exocytosis is believed to occur (Holdaway-Clarke and Hepler, 2003). Cell wall yielding then allows turgor-induced stretching and stimulates the flow of water into the cell, down its potential gradient. During oscillatory growth, enzymes that mediate wall yielding are believed to be periodically activated and inhibited, and this is thought to drive periodicity in wall loosening and growth.

The polymers required for synthesis and growth of the pollen tube cell wall are delivered by secretion in the subapical growth zone. The cell wall in the apical dome and subapical growth zone is essentially exclusively composed of primarily methylesterified pectins; unesterified pectins and the main load-bearing component of primary cell walls, the cellulose–hemicellulose framework, are located in distal regions (Taylor and Hepler, 1997; Bosch and Hepler, 2005; Parre and Geitmann, 2005). The pectin mesh is thought to be largely independent of and without extensive covalent cross-links to the cellulose–hemicellulose

framework, irrespective of the degree of pectin methylesterification (Carpita and Gibeaut, 1993; Cosgrove, 2005). Pectins are secreted as jelly-like polymers in the subapical growth zone in a highly methylesterified form, with ~70–80% of GalA residues methylesterified and 20–30% of residues unesterified. Newly secreted pectins are recognized by the JIM7 antibody that binds to at least one methylesterified GalA adjacent to one unesterified GalA (Bosch and Hepler, 2005). Highly unesterified pectins are identified by recognition with the JIM5 antibody that binds to at least four contiguous unesterified GalA residues (Bosch and Hepler, 2005). Pectins in the distal pollen tube are highly unesterified. The higher the degree of de-esterification, the more rigid the pectin gel becomes due to extensive cross-linking with cations such as Ca^{2+}.

Pectin de-esterification is catalysed by pectin methylesterase (PME) (Carpita and Gibeaut, 1993; Bosch and Hepler, 2005, 2006; Bosch *et al.*, 2005; Pelloux *et al.*, 2007; Rockel *et al.*, 2008). In *Arabidopsis* pollen tubes, PME was localized at the cell wall along the entire pollen tube including the apical dome, while an inhibitor of PME (PMEI) was localized primarily along the apical dome and in the subapex (Rockel *et al.*, 2008). This differential spatial localization pattern can explain why PME activity is excluded from the apex and highly unesterified pectins are localized to distal regions of pollen tubes. Subcellular localization patterns of membrane proteins are determined by the site of exocytosis (Zonia and Munnik, 2009). PMEI localization along the apical dome probably results from exocytic insertion in the mid-zone and near the leading edge of the growth zone followed by anterograde movement to the apex (Zonia and Munnik, 2009). PMEI was found in FYVE-induced endosomal aggregates (Rockel *et al.*, 2008), consistent with endocytic uptake at the apex

Detailed biochemical knowledge of the pectin mesh structure at the pollen tube apex is lacking. It is known that turgor pressure provides the energy to drive incorporation of secreted pectins into the cell wall of *Chara* (Proseus and Boyer, 2005, 2006), and this is considered to be generally true for plant cells (Cosgrove, 2005) (Table 1). Turgor pressure in the apical region of tobacco pollen tubes is highest just before the start of the growth cycle, thus supporting a temporal correlation between hydrodynamics and cell wall deposition (Fig. 2). Newly secreted pectins diffuse some distance in the wall during turgor-induced wall stretching, indicating that their position is not immediately fixed and the existing cell wall tolerates significant stretching, pliancy, and porosity (Cosgrove, 2005, and references therein). Integration of these immature cell wall materials into the existing wall is initially mediated by physical interactions (Carpita and Gibeaut, 1993; Cosgrove, 2005). The pectin chains condense by cross-linking with Ca^{2+} to form junction zones linking two antiparallel chains. The number of contiguous unesterified GalA residues required to form a stable junction *in muro* is unknown. At very low Ca^{2+} concentrations, it is thought that two chains can form a stable junction when the overlap is ~14 GalA residues; if sufficient Ca^{2+} is present, some interrupting esterified GalA can be tolerated and still form a stable junction zone; if the Ca^{2+} concentration is high, formation of stable junctions is prevalent and four-chain or higher order stacking of chains into multiple eggbox structures occurs (Carpita and Gibeaut, 1993, and references therein). These studies suggest that the cell wall in the expanding subapical growth zone is composed of a dynamically changing population of newly secreted pectins and condensed pectin chains. The cell wall distal to the growth zone would rapidly become a rigid gel due to the activity of PME, while the cell wall anterior to the growth zone would retain more pliancy even as the population of pectin chains and stacked chains continued to accumulate.

Biochemical modification (cell wall loosening) of the wall in the distal pollen tube is likely to be required during synthesis and assembly of the cellulose–hemicellulose framework embedded in the rigid unesterified pectin matrix. However, it is probable that modification of the existing pectin mesh in the actively expanding subapical growth zone is not required for the secretion and deposition of new pectins (Cosgrove, 2005; Proseus and Boyer, 2005, 2006), or for the condensation and assembly of new pectin chains (Carpita and Gibeaut, 1993; Cosgrove, 2005). Thus, cell wall synthesis in the expanding growth zone would be self-organized by physical processes dependent on apical osmotic pressure, which drives incorporation of newly secreted pectins into the wall (Proseus and Boyer, 2005, 2006) and stretches the existing wall to enable diffusion, self-assembly, and integration of the newly secreted wall materials into the apical pectin mesh (Carpita and Gibeaut, 1993; Cosgrove, 2005) (Fig. 2). Apical volume and pressure oscillate during oscillatory pollen tube growth, and this leads to periodicity in disruption of the cell surface in the actively expanding growth zone (Zonia and Munnik, 2008*b*), probably due to periodicity in the secretion, diffusion, deposition, and assembly of newly delivered pectins. Support for this was recently obtained by observations of periodicity in accumulation of pectin wall material along the apical dome during oscillatory growth of tobacco and lily pollen tubes (McKenna *et al.*, 2009). Additionally, experimentally induced relaxation of the cell wall using PME or auxin leads to changes in growth rate oscillation frequencies (Zerzour *et al.*, 2009).

Cytoskeleton and cell stiffness during swelling and growth

Cytoskeletal arrays have central roles in a number of processes that are crucial for plant cell growth and function, including cytoplasmic streaming and intracellular transport, exocytosis and endocytosis, and cell division and morphogenesis (Staiger, 2000; Wasteneys and Galway, 2003; Smith and Oppenheimer, 2005; Staiger and Blanchoin, 2006; Hussey *et al.*, 2006; Lovy-Wheeler *et al.*, 2007; Cai and Cresti, 2009). The architectures of cytoskeletal polymers have been studied extensively in pollen tubes, and continual advances in methods to label actin and microtubule arrays in live cells are refining our understanding of their regulation and

dynamics (Cheung *et al.*, 2008; Era *et al.*, 2009; Vidali *et al.*, 2009). Actin polymers are organized as long filaments or bundled filaments with an axial organization along the pollen tube from the base to the subapex, where they become organized into a short and dense fringe structure (Lovy-Wheeler *et al.*, 2005; Vidali et al, 2009; Cheung *et al.*, 2008). Very fine actin filaments periodically extend between the subapical fringe and the apex (Fu *et al.*, 2001). In pollen tubes undergoing oscillatory growth, the appearance of very fine filaments near the apex correlates with the phase of minimum growth rate and reaches a maximum before the growth peak (Fu *et al.*, 2001; Lee *et al.*, 2008*c*). Exocytic vesicles are trafficked along actin filaments by myosin motors and delivered to the site of exocytosis in the subapical growth zone via the actin fringe. Microtubule arrays and their associated kinesin motors are known to be important for trafficking of organelles (Romagnoli *et al.*, 2003, 2007; Cai and Cresti, 2009), and for the organization and synthesis of the cellulose–hemicellulose network of the plant cell wall (Lloyd and Chan, 2008; Crowell *et al.*, 2009).

The properties of the solid phase of the cytoplasm are determined and regulated to a large extent by actin architecture and dynamics. Actin polymers display features of an active gel with non-linear mechanical properties of both particles and stiff rods, and in live cells they are far from equilibrium (Bausch and Kroy, 2006). Extensive reorganization of the actin cytoskeleton occurs rapidly in response to osmotic perturbations in most vertebrate cell types (Spagnoli *et al.*, 2008; Hoffmann *et al.*, 2009, and references therein). Cell swelling induces a net decrease in actin polymerization, while cell shrinking induces a net increase (Table 1). These properties are consistent with observations of actin arrays in the apical dome during oscillatory pollen tube growth. Closer examination of a range of cell types under differing conditions revealed that during cell swelling or when subjected to a transient sheer force, the actin cytoskeleton fluidizes in a manner that marks it as a universal response (Trepat *et al.*, 2007; Spagnoli *et al.*, 2008). A single unifying relationship emerged in which the closer the system was to a solid-like state before application of a transient force, the greater the extent of fluidization and the faster subsequent resolidification. This behaviour is consistent with soft glass rheology. Many cell types are known to have cytoplasmic glasses and display glassy phase transitions, including *Typha latifolia* pollen (Buitink *et al.*, 1998, 2000). The end result is counterintuitive but consistent with glass rheology, in that cells become softer when subjected to transient force, as a result of fluidization of the cytoplasmic glass (Trepat *et al.*, 2007). Similarly, when subjected to osmotic swelling, cells become softer and not stiffer as measured by atomic force microscopy (Spagnoli *et al.*, 2008). A recent extension of this work conclusively demonstrated that hyperosmotic treatment inducing hydrodynamic efflux and cell shrinking causes cells to become stiffer (Zhou *et al.*, 2009). Thus, the combined evidence on cell properties following hydrodynamic treatment distils to a universal response where cells become softer as they swell and stiffer as they shrink (Trepat *et al.*, 2007; Spagnoli *et al.*, 2008; Zhou *et al.*, 2009).

These results have interesting ramifications for pollen tubes during oscillatory growth. A comparison of apical volume oscillations with oscillations in actin filament organization at the apex reveals that actin filaments extend near to the apex when the apical volume is minimal (temporal correspondence to the phase of minimum growth), while actin filaments are absent from the apex when the apical volume is maximal (temporal correspondence to the phase of maximum growth) (Fig. 3). Thus, due to cell geometry, vectorial hydrodynamic flow, and properties of the cytoskeleton, turgor-induced elongation of the tip may be mediated by a spatially localized pressure increase only at the apex that causes localized expansion of the plasma membrane proximal to the fine actin filaments near the apex, in a process similar to cell blebbing (Fig. 3). This further suggests that dynamic reorganization of the actin architecture at the pollen tube apex may be mediated at least in part by physical forces in addition to the known actin-binding proteins and regulators that modulate actin polymerization and depolymerization. Fluidization of the cytoplasm during the phase of maximally increasing osmotic pressure would also serve as a safety valve that ensures sufficient increase in pressure to drive growth but buffers the apical plasma membrane and pectin wall from a catastrophic pressure increase by redistribution of excess pressure into the cytoskeletal network.

A recent study used a micro-indenter to probe mechanically the relative stiffness/compliance of growing pollen tubes and reports some evidence to suggest that the subapical growth zone becomes more compliant before the start of the growth cycle (Zerzour *et al.*, 2009), which corresponds to a phase with increased osmotic swelling and is thus consistent with the universal response. However, the data also show evidence of a transient increase in stiffness just at the start of the growth cycle, which corresponds to a phase with decreased volume and apical shrinking, and is also consistent with the universal response. Crucial controls to measure relative stiffness and compliance in osmotically swollen and shrunken pollen tubes were lacking. Thus, it is not yet clear whether the measured changes in stiffness or compliance in the pollen tube subapex reflect the appearance and assembly of newly secreted immature wall materials in the subapical growth zone, fluidization of the cytoskeleton during apical volume increase or transient force, or stiffening of the apical region during hydrodynamic efflux and apical shrinking.

Phosphoinositide, phosphatidic acid, and inositol polyphosphate signalling

Phospholipids and their metabolites are a major class of cellular signals that are involved in ion channel regulation, cytoskeletal organization, membrane trafficking, and membrane architecture. The biochemistry and functional roles of phospholipid signalling in plants have been addressed in

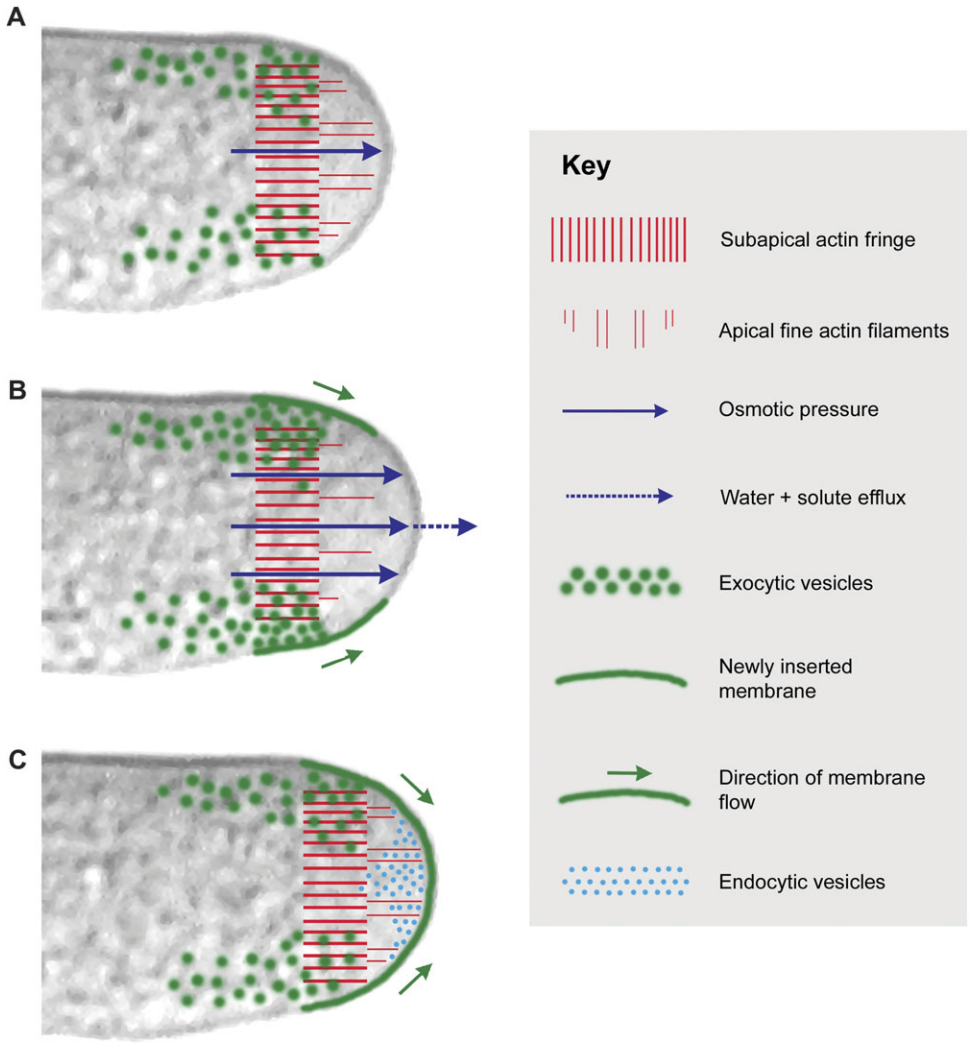

Fig. 3. The hydrodynamic model of pollen tube growth. For simplicity, only eight elements of the entire growth network are depicted as defined in the key. Temporal and spatial asymmetries in osmotic pressure are proposed to drive pollen tube growth in a process with similarities to blebbing. (A) Intermediate osmotic pressure. Mass flow and hydraulic conductivity establish vectorial hydrodynamic flux toward the apex. Increasing osmotic pressure in the apical region will differentially exert localized pressure effects depending on plasma membrane attachment to the local cytoplasmic architecture. The apical dome presents an osmotic sink due to exclusion of larger vesicles, organelles, endomembrane networks, and extensively bundled actin cables, and due to hydraulic and elastic contraction of the rest of the cytoplasm. (B) High osmotic pressure. Increasing pressure on the apical plasma membrane will exploit any weak point, force it to bulge outward, and detach the local area from any underlying cytoplasmic architectures. The entire local area then becomes a weak point for further membrane detachment, enabling increased hydrodynamic flow and stretching of the membrane. The increased stretch and flow elongate the newly inserted membrane in the growth zone toward the apex. (C) Low osmotic pressure after hydrodynamic unloading at the apex and apical volume decrease. Newly inserted membrane flows toward the apex where it is internalized for recycling by smooth vesicle endocytosis. The actin networks comprising the subapical fringe undergo an anterograde shift; nothing is known about how this occurs but it probably involves net polymerization. There is a net increase (net polymerization) in apical fine actin filaments and these extend between the subapical fringe and the apical plasma membrane. Endocytic and endosomal trafficking undergo increases during this phase. For simplicity, endocytosis at the apex is depicted only at this phase (C), but experimental evidence shows that it is essentially constitutive.

detail in recent reviews (Meijer and Munnik, 2003; Testerink and Munnik, 2005; Zonia and Munnik, 2006; Thole and Nielsen, 2008; Heilmann, 2009; Munnik and Testerink, 2009; Xue *et al.*, 2009; Munnik and Vermeer, 2010). Recent work with fluorescent biosensors has revealed detailed information about the subcellular localization of specific components involved in plant phospholipid signalling. Correlation of these results with previous biochemical analyses enables a broader understanding of signalling cascades in pollen tube growth and function, and has identified four major hubs (including metabolic precursors and products) that revolve around phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], phosphatidic acid (PtdOH), phosphatidylinositol 3-phosphate (PtdIns3P), and the soluble inositol

polyphosphates. This section will focus on spatiotemporal dynamics of these lipid signals in light of new data on vesicle trafficking and cell hydrodynamics in pollen tube growth.

PtdIns(4,5)P_2 is involved in clathrin-mediated endocytosis (Brearley, 2008; Konig *et al.*, 2008; Botelho, 2009) and reorganization of the actin cytoskeleton (Staiger, 2000; Meijer and Munnik, 2003; Staiger and Blanchoin, 2006). PtdIns(4,5)P_2 is synthesized via phosphorylation of PtdIns4P by phosphatidylinositol-4-phosphate 5-kinase (PI4P5K), and its hydrolysis by phospholipase C (PLC) produces diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. PtdIns(4,5)P_2 has a rapid turnover rate in growing tobacco pollen tubes (Zonia and Munnik, 2004). A biosensor specific for PtdIns(4,5)P_2 (YFP:$PH_{PLC\delta1}$) localizes to the plasma membrane in the apex, apical dome, and subapex (Kost *et al.*, 1999; Helling *et al.*, 2006). In root hairs, a probe for PtdIns4P (YFP:PH_{FAPP1}) is localized on motile punctate structures that label with a Golgi probe, and also has a gradient of localization along the plasma membrane that is highest in the apical dome and subapex (Vermeer *et al.*, 2009). Fluorescent constructs with PIP5K4 (PIP5K4:GFP) and PIP5K5 (PIP5K5:EYFP) show strong localization in the subapex of tobacco and *Arabidopsis* pollen tubes, with diminished localization along the flanks of the apical dome (Sousa *et al.*, 2008; Ischebeck *et al.*, 2008). PLC3 (PLC3:YFP) from tobacco and PLC1 (GFP:PLC1) from petunia show prominent accumulation along the subapex and distal plasma membrane in tobacco and petunia pollen tubes, respectively (Dowd *et al.*, 2006; Helling *et al.*, 2006). DAG (Cys1:YFP) accumulates at the plasma membrane in tobacco pollen tubes similarly to PtdIns(4,5)P_2, along the apical dome and subapex, but also accumulates in the distal tube (Helling *et al.*, 2006).

The results from mutant analyses, inhibitor effects, and localization studies of PtdIns(4,5)P_2 are easily interpreted within the framework of the new model for pollen tube growth. Biosynthesis of PtdIns(4,5)P_2 appears to occur at the plasma membrane via PIP5K phosphorylation of PtdIns4P, which is thought to mediate trafficking of exocytic vesicles from the *trans*-Golgi complex to the plasma membrane (Thole and Nielsen, 2008; Heilmann, 2009). Exocytic vesicles incorporate into the plasma membrane in the subapex, thus formation of PtdIns(4,5)P_2 would occur in this region. Anterograde membrane flow from the subapex to the apex would lead to the observed distribution of PtdIns(4,5)P_2 in the subapex and along the apical dome. A low level of PtdIns(4,5)P_2 retrograde flow from the subapex is a substrate pool for PLC hydrolysis, as PLC localizes along the distal membrane and to a reduced degree in the subapex, and thus extensive spreading of PtdIns(4,5)P_2 in distal regions is prevented. DAG is small and mobile, and readily diffuses through the membrane, thus its localization both anterograde and retrograde to the subapex (Helling *et al.*, 2006). Blocking PLC activity caused spreading of PtdIns(4,5)P_2 from the apical region to the distal tube, and inhibited the accumulation of DAG (Helling *et al.*, 2006), results that are consistent with the new growth model. Fluorescence recovery after photobleaching (FRAP) of the lateral plasma membrane distally adjacent to the subapex showed that PLC1 undergoes retrograde flow from the subapex (Dowd *et al.*, 2006), further supporting the new model.. The same study performed FRAP of the area near the apical dome that includes the growth zone and the pool of endosomal vesicles, and observed recovery within 5–10 s; however, this could reflect recovery of exocytic and/or endocytic pathways (Dowd *et al.*, 2006). The *pip5k4* null mutants have reduced endocytic uptake and membrane recycling in tobacco pollen tubes (Sousa *et al.*, 2008), supporting a role for apical PtdIns(4,5)P_2 in endocytosis. Overexpression of either PIP5K4 or PIP5K5 in *Arabidopsis* pollen tubes caused an increase and mislocalization of PtdIns(4,5)P_2 (RedStar:$PH_{PLC\delta1}$), and induced tip branching and increased accumulation of pectin (Ischebeck *et al.*, 2008). This highlights the importance of localization of PtdIns(4,5)P_2 for correct signalling during pollen tube growth.

The second major hub of signalling revolves around PtdOH. PtdOH can be synthesized by the PLC and/or PLD signalling pathways: via PLC by phosphorylation of DAG by diacylglycerol kinase (DGK), or via PLD by hydrolysis of structural lipids such as phosphatidylcholine or phosphatidylethanolamine (Meijer and Munnik, 2003; Wang, 2004; Testerink and Munnik, 2005; Zonia and Munnik, 2006; Arisz *et al.*, 2009; Li *et al.*, 2009; Munnik and Testerink, 2009). PtdOH signalling is important for pollen tube growth and is proposed to have roles in membrane architecture and trafficking (Zonia and Munnik, 2004, 2009; Monteiro *et al.*, 2005*a*, *b*). PtdOH formation has a profound effect on membrane curvature, surface charge, and protein interaction (Kooijman *et al.*, 2003, 2005, 2007, 2009; Kooijman and Burger, 2009). In animal cells, PtdOH is proposed to have a biophysical role in generating negative membrane curvature to promote both exocytosis and endocytosis (Bader and Vitale, 2009; Donaldson, 2009), and PtdOH and PLD are linked with the regulation of PI4P5K and organization of the actin cytoskeleton (Cockcroft, 2009; Rudge and Wakelam, 2009). In plant cells, PtdOH has recently been shown to bind actin-capping protein, which stimulates the uncapping of actin filament barbed ends and promotes actin filament assembly (Huang *et al.*, 2006; Staiger and Blanchoin, 2006). PtdOH is phosphorylated by phosphatidic acid kinase (PAK) to produce diacylglycerol pyrophosphate (DGPP). Localization studies show that DAG accumulates along the entire apical dome, the subapex, and distal regions (Helling *et al.*, 2006), indicating that it undergoes lateral diffusion through the plasma membrane from the site of its biosynthesis in the subapex (Zonia and Munnik, 2008*a*, 2009). This represents an abundant substrate pool for spatiotemporal activation of DGK. In growing tobacco pollen tubes, PtdOH has a very rapid turnover rate and high level of accumulation, and DGPP turnover and accumulation are similar to those of PtdOH but at a reduced level (Zonia and Munnik, 2004). The evidence in tobacco and *Agapanthus* pollen tubes indicates that PtdOH results from activation of both PLD and PLC pathways (Zonia and Munnik, 2004; Monteiro

et al., 2005*a*). This could function as a tight cross-link to integrate signalling from both pathways.

Less research has been invested to date in the third signalling hub that revolves around PtdIns3P. PtdIns3P is produced via phosphorylation of PtdIns by PI3K. PI3P5K subsequently phosphorylates PtdIns3P to produce PtdIns(3,5)P_2. *Arabidopsis* contains a single PI3K (AtVPS34) and knockout mutations are lethal, indicating the importance of this signalling pathway (Welters *et al.*, 1994; Lee *et al.*, 2008*b*). Two putative PI3P5K homologues have been identified in *Arabidopsis*, FAB1A and FAB1B (Mueller-Rober and Pical, 2002; Whitley *et al.*, 2009). Double mutant *fab1a*/*fab1b* lines are pollen lethal, while single mutants have severe defects in vacuolar organization in pollen microspores (Whitley *et al.*, 2009). In yeast, PtdIns3P and PtdIns(3,5)P_2 are involved in membrane trafficking through the late endosomal pathway to the vacuole (Michell *et al.*, 2006; Dove *et al.*, 2009). Fluorescent biosensor constructs with a domain that specifically binds PtdIns3P, called FYVE, have been used to probe plant cells. In BY-2 cells, YFP:2XFYVE localizes on late endosomes and the pre-vacuolar compartment; in guard cells and leaf epidermal cells of *Arabidopsis* it localizes on late endosomes and the vacuolar membrane (Vermeer *et al.*, 2006). In *Arabidopsis* root hairs, GFP:2XFYVE localizes on large particles that appear to be late endosomes (Lee *et al.*, 2008*a*). In tobacco pollen tubes, mRFP:FYVE labelled large aggregates, consistent with endosomal/vacuolar trafficking (Rockel *et al.*, 2008).

Inositol polyphosphate signals have demonstrated roles in ion channel regulation, ion fluxes, membrane trafficking, and mRNA export from the nucleus (Irvine and Schell, 2001; Munnik and Vermeer, 2010). There are up to 60 different inositol polyphosphate isomers, but the function of only a few of them is presently known. The classic signal in mammalian cells, Ins(1,4,5)P_3, is involved in mobilizing free Ca^{2+} from intracellular stores following receptor-mediated hydrolysis of PtdIns(4,5)P_2 to Ins(1,4,5)P_3 and DAG (Irvine, 2003). However, in yeast cells, the evidence indicates that Ins(1,4,5)P_3 is further phosphorylated by inositol phosphate kinases (IPK1 and IPK2) to InsP_4, InsP_5, and InsP_6 (inositol hexakisphosphate, also called phytic acid), and it is the InsP_6 product of InsP_3 metabolism that functions as the signal involved in mobilizing mRNA export from the nucleus (York *et al.*, 1999; Ives *et al.*, 2000; Perera *et al.*, 2004; York, 2006). IPK2 has a demonstrated role in pollen tube growth, with antisense lines undergoing increased growth under non-optimal low Ca^{2+} concentrations (Xu *et al.*, 2005). In guard cells, InsP_6 mobilizes cytosolic free Ca^{2+} from endomembrane stores (Lemtiri-Chlieh *et al.*, 2003) and specifically inhibits an inward rectifying K^+ channel ($I_{K,in}$) in a Ca^{2+}-dependent manner (Lemtiri-Chlieh *et al.*, 2000). These studies showed that InsP_6 was ~100-fold more potent than Ins(1,4,5)P_3 in inhibiting $I_{K,in.}$ In pollen tubes, previous studies have documented a time-lagged increase in free Ca^{2+} after release of caged Ins(1,4,5)P_3 (Franklin-Tong *et al.*, 1996; Malho, 1998). Maximal free Ca^{2+} increases were detected during ~3.5–10 min after Ins(1,4,5)P_3 release (Franklin-Tong *et al.*, 1996). This suggests that microinjected Ins(1,4,5)P_3 might be further phosphorylated, and that higher order inositol polyphosphates such as InsP_6 might function to mediate Ca^{2+} mobilization as shown in guard cells.

A second inositol polyphosphate with a specific role in tobacco pollen tube growth is Ins(3,4,5,6)P_4 (Zonia *et al.*, 2002). Current evidence indicates that Ins(3,4,5,6)P_4 synthesis is linked to Ins(1,3,4,5,6)P_5 by a reversible phosphatase/kinase cascade (Shears, 1998; Brearley and Hanke, 2000; Irvine and Schell, 2001). The function of Ins(3,4,5,6)P_4 was first identified in epithelial cells, where it specifically inhibits a Cl^- channel that is involved in maintenance of cell volume and secretion (Shears, 1998; Ho *et al.*, 2001; Rudolf *et al.*, 2003; Mitchell *et al.*, 2008). Cl^- channels have crucial roles in plant cell osmoregulation (Teodoro *et al.*, 1998; Shabala *et al.*, 2000; White and Broadley, 2001). In tobacco pollen tubes, microinjected Ins(3,4,5,6)P_4 specifically inhibits Cl^- efflux from the apex, induces massive swelling of the apical region, and ultimately inhibits pollen tube growth (Zonia *et al.*, 2002). It is proposed that Ins(3,4,5,6)P_4 has a role in regulating cell hydrodynamics, osmotic pressure, and apical volume during pollen tube growth by its inhibition of anion efflux from the apex (Zonia and Munnik, 2006, 2007).

PtdIns(4,5)P_2 levels rapidly increase in response to hyperosmotic cell volume decrease in a variety of cell types (Hoffman *et al.*, 2009) (Table 1). PtdIns(4,5)P_2 levels also increased rapidly following hyperosmosis in tobacco pollen tubes (Zonia and Munnik, 2004) (Table 1) (Fig. 2). The increased PtdIns(4,5)P_2 will have profound effects on membrane trafficking and actin cytoskeletal organization. As discussed in previous sections, hyperosmosis increases endocytosis and is predicted to promote a net increase in actin polymerization, temporally consistent with the phase of minimum growth. Levels of PtdIns(3,5)P_2 also increase in response to hyperosmosis (Zonia and Munnik, 2004) (Table 1) (Fig. 2). D-3-Polyphosphoinositides are involved in endosomal trafficking, and thus could have a crucial role in managing the increased flux through endocytic/endosomal pathways in response to hyperosmotically induced cell shrinking.

Hypo-osmotic cell swelling activates PLD in mammalian cells (Tomassen *et al.*, 2004; Hoffmann *et al.*, 2009) and induces increased PtdOH in the unicellular green alga *Dunaliella salina* (Einspahr *et al.*, 1988) (Table 1). In pollen tubes, hypo-osmotic cell swelling activates PLD and increases PtdOH levels within 30 s (Zonia and Munnik, 2004) (Fig. 2). Activation of the PLD pathway potentiates Cl^- secretion in mammalian cells (Vajanaphanich *et al.*, 1993; Oprins *et al.*, 2001, 2002), which drives water efflux and regulatory cell volume decrease. Increased PtdOH promotes negative curvature of the plasma membrane, which facilitates exocytic vesicle fusion and secretion (Kooijman *et al.*, 2003, 2005; Bader and Vitale, 2009; Kooijman and Burger, 2009). This supports the temporal correlation of PtdOH increase, Cl^- secretion, exocytosis, and growth (Fig. 2). Additionally, increased PtdOH could promote actin assembly at the barbed ends by binding to actin-capping protein (Huang *et al.*, 2006; Staiger and Blanchoin, 2006),

which could facilitate spatiotemporal synchronization in the elongation of actin filaments and the actin fringe in conjunction with cell elongation and growth.

Hyperosmotic stress has been shown to induce increases in Ins(1,4,5)P_3 in carrot suspension cells and *Arabidopsis* cultures, seedlings, and leaves (Drobak and Watkins, 2000; DeWald *et al.*, 2001; Takahashi *et al.*, 2001; Im *et al.*, 2007; Konig *et al.*, 2007) (Table 1). As discussed above, evidence is emerging that Ins(1,4,5)P_3 is phosphorylated to $InsP_4$, $InsP_5$, and $InsP_6$. Thus, Ins(3,4,5,6)P_4 levels might increase with increasing $InsP_5$ (because it is linked through a reversible phosphatase/kinase cascade), and this would inhibit anion efflux, consistent with the hydrodynamics model of pollen tube growth. Similarly, increased $InsP_6$ might mobilize free Ca^{2+} that would affect ion channels and endocytosis.

Ion fluxes in pollen tube growth

Ion flux across the plasma membrane to regulate cell osmotic potential and volume is postulated to be the most ancient signal transduction pathway that arose with the onset of cellular life, and became refined during the proliferation of cellular complexity (Martinac, 2004; Kung, 2005). Ion flux is the most rapid response to changes in osmotic potential across the plasma membrane in a wide variety of cell types from bacteria to eukaryotes and plants (Blatt, 2000; Schroeder *et al.*, 2001; White and Broadley, 2001; Pandey *et al.*, 2007; Martinac *et al.*, 2008; Hoffmann *et al.*, 2009) (Table 1). Ion flux functions as an osmotic pressure safety valve and to drive water into or out of the cell.

Ion fluxes (Ca^{2+}, K^+, H^+, and Cl^-) have been intensively studied in pollen tubes, and they are crucial for growth (Holdaway-Clarke and Hepler, 2003). Growing pollen tubes have an intracellular free Ca^{2+} gradient that is highest in the apical dome, an acidic region at the tip, and an alkaline band near the subapex that corresponds to the location of the growth zone. In growing pollen tubes, cations (Ca^{2+}, K^+, and H^+) display net influx at the apex while Cl^- anion effluxes from the apex. During oscillatory growth, all ion fluxes oscillate with the same frequency as growth oscillations, but have phase shifts with respect to the growth cycle (Fig. 2). The intracellular Ca^{2+} gradient peaks after peak growth, with a phase shift of ~30 °. Maximum Ca^{2+} and K^+/H^+ influxes at the apex occur after peak growth, with phase shifts of ~100 ° and 130 °, respectively. Anion efflux from the apex occurs in phase with growth. Disruption of Ca^{2+} or Cl^- fluxes leads to apical swelling and growth arrest (Holdaway-Clarke and Hepler, 2003; Breygina *et al.*, 2009). The new model considers that ion fluxes in pollen tubes are involved in regulating and responding to changes in cell hydrodynamics in the apical region, and are essential elements in regulatory volume decrease and regulatory volume increase (Zonia and Munnik, 2007, 2009).

Aside from their role in electrophysiology and cell osmotic potential, ions also interact with and potentially regulate a variety of cellular targets including the actin cytoskeleton, membrane transporters, protein kinases, small GTPases, exocytosis and endocytosis, and cell wall pectins. A discussion of these results is beyond the scope of this review, and interested readers are referred to a previous review (Holdaway-Clarke and Hepler, 2003). The current review will briefly mention possible roles of ions in regulation of exocytosis and endocytosis. Earlier work demonstrated a role for Ca^{2+} in plant cell exocytosis (Zorec and Tester, 1992; Thiel and Battey, 1998; Battey *et al.*, 1999). Thus, the tip high Ca^{2+} gradient and Ca^{2+} influx at the tip suggested that Ca^{2+} is involved in regulating exocytosis at the tip. However, recent data indicate that smooth vesicle endocytosis occurs at the tip and exocytosis occurs in the subapex (Moscatelli *et al.*, 2007; Bove *et al.*, 2008; Zonia and Munnik, 2008*a*, *b*, 2009). In light of this, recent work on neurons may hold some clues. At axonal synaptic terminals, Ca^{2+} is involved in both exocytosis and endocytosis (He *et al.*, 2008; Idone *et al.*, 2008; Jackson and Chapman, 2008; Dittman and Ryan, 2009). High Cl^- levels selectively inhibit a form of endocytosis that is called 'kiss-and-run' exocytosis or flicker fusion (Smith *et al.*, 2008). The endocytosis rate appears to be linked to the rate of exocytosis, and these are sensitive to levels of Ca^{2+} and Cl^-. In tobacco pollen tubes, an integrated balance between rates of exocytosis, endocytosis, and growth is similarly observed during osmotic forcing of increased growth (hypo-osmosis) versus decreased growth (hyperosmosis) (Zonia and Munnik, 2008*a*) (Fig. 2). High Cl^- levels blocked tobacco pollen tube growth, but did not immediately block smooth vesicle endocytosis at the apex (Breygina *et al.*, 2009), suggesting that this endosomal pathway differs from the 'kiss-and-run' exocytosis/endocytosis pathway that has been suggested to occur at the pollen tube apex (Samaj *et al.*, 2006; Bove *et al.*, 2008).

Small G-proteins in pollen tube growth: Rho/Rac

Small GTPases have crucial roles in pollen tube growth and have been the focus of intensive research (Nibau *et al.*, 2006; Samaj *et al.*, 2006; Yang and Fu, 2007; Kost, 2008; Lee and Yang, 2008; Nielsen *et al.*, 2008; Woollard and Moore, 2008; Yalovsky *et al.*, 2008). They have roles in mediating actin cytoskeletal reorganization, Ca^{2+} flux, reactive oxygen species, exocytosis, endocytosis and endosomal trafficking, and phospholipid signalling. An understanding of their exact function in these processes is complicated by the large number of upstream regulators, only a few of which have been identified to date—guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs)—that are themselves subject to regulation by upstream signalling pathways. The current review is limited to brief mention of the role of Rho GTPases in endocytosis and potential regulation of small G-proteins by cell hydrodynamics.

ROP1 (Rho of plants) is localized along the plasma membrane in the apical dome of growing pollen tubes, and its activity appears to be high before the start of the growth

cycle (Fig. 2) (Lee *et al.*, 2008*c*). Because of this localization, and because the apex was thought to be the site of exocytosis, ROP1 was considered to function in mediating exocytosis. However, there is extensive evidence indicating Rho GTPase function in endocytosis and endocytic trafficking, as well as exocytosis (Ridley, 2001; Qualmann and Mellor, 2003). Recent work has shown that ectopic expression of activated Rho/Rac in *Arabidopsis* root hairs disrupts endocytic uptake of FM 4-64 (Bloch *et al.*, 2005; Yalovsky *et al.*, 2008). Another study shows that expression of constitutively active ROP in guard cells attenuates light-induced stomatal opening (Jeon *et al.*, 2008). Light-induced stomatal opening occurs by hydrodynamically driven swelling and increased exocytosis, while stomatal closure occurs by hydrodynamically driven shrinking and increased endocytosis. Thus, attenuation of stomatal opening by constitutively active ROP might be due to its activation of endocytosis and interference with exocytosis. Further work will be required to clarify the function of ROP1 in plant cells and in pollen tube growth.

Rho GTPase activity is rapidly increased after hypertonic cell shrinking in epithelial cells, and decreased in osmotically swollen cells (Hoffmann *et al.*, 2009). In *Arabidopsis* seedlings, salt and drought stress activated several family members of ROP-GEFs (Shin *et al.*, 2009). In tobacco pollen tubes, GFP–RIC4 (a ROP-interacting protein that activates ROP) accumulates at the plasma membrane along the apical dome before the start of the growth pulse (Hwang *et al.*, 2005), and this has been correlated with increased ROP activity (Yang and Fu, 2007; Lee and Yang, 2008). Further work will be required to investigate possible stimulation of ROP activity by hypertonic treatment or apical volume decrease.

Fertilization and self-incompatibility

The first stage of pollen growth *in planta* is hydration and germination on the stigma. The last stage is arrival of the pollen tube at the embryo sac, penetration of the synergid cells, and rupture of the tip to discharge the sperm cells and effect fertilization. Both of these stages are critically dependent on cell hydrodynamics (Table 1). Lipids on the stigmatic surface (for species with wet and dry stigmas) and proteins in the pollen coat are required for controlling the directional flow of water to pollen to enable hydration, which is requisite for pollen tube germination and growth (Lush *et al.*, 1998; Wolters-Arts *et al.*, 1998; Updegraff *et al.*, 2009). Upon reaching the micropyle, the pollen tube ceases growth and the apical region undergoes a massive increase in volume and pressure that induces explosive bursting near the apex. Initial rates of discharged material are estimated to be of the order of 10 000 $\mu m^3\ s^{-1}$ during the first 0.1 s for *Torenia* pollen tubes (Higashiyama *et al.*, 2000).

Growth arrest and hydrodynamically induced ramping of pressure in the pollen tube apical region prior to sperm cell discharge are under control of both female and male genes. FERONIA encodes a receptor-like kinase expressed on the synergid cell surface, and upon reaching *feronia/sirene* embryo sacs pollen tubes continue growing and fail to discharge (Huck *et al.*, 2003; Rotman *et al.*, 2003; Escobar-Restrepo *et al.*, 2007). LORELEI encodes a putative glucosylphosphatidylinositol-anchored protein expressed in the synergid cells, and similar to *feronia/sirene*, pollen tubes fail to cease growth and discharge upon reaching *lorelei* embryo sacs (Capron *et al.*, 2008). The pollen-expressed homologues of FERONIA (ANXUR1 and ANXUR2) have recently been identified in *Arabidopsis* (Boisson-Dernier *et al.*, 2009; Miyazaki *et al.*, 2009). ANXUR1/2 encode RLKs that localize to the pollen tube apical region, with significant accumulation in the subapex and near the apical dome. Single mutants lack negative effects on phenotype, but double mutants have severely swollen tubes and spontaneously discharge *in vitro*. However, double mutants germinate normally on the stigma and grow through the papillar cell layer, but most discharge in the style or between the stigma and style.

Self-incompatibility (SI) is a highly resource-intensive system that plants have developed to prevent inbreeding. Several families have invested in SI systems that specifically target pollen hydrodynamics (Nasrallah, 2005, and references therein). In self-incompatible members of the crucifer family (eg. *Brassica*, *Arabidopsis lyrata*) and in the grass family, SI blocks hydration of self-pollen grains within minutes after pollen arrival on the stigmatic surface, whereas non-self-pollen grains undergo hydration and increase cell volume by 2- to 3-fold (Dickinson, 1995). The SI response is triggered by interaction of the S-locus receptor kinase on the stigmatic surface with S-locus cysteine-rich proteins located in the pollen coat, functions to isolate self-pollen hydrodynamically, and enables single papillar cells simultaneously to accept non-self-pollen and reject self-pollen. The rejected pollen grains fail to hydrate and subsequently fail to germinate and form a pollen tube.

Conclusions and perspectives

This review has proposed a spatiotemporal model of signalling networks regulating pollen tube growth that is integrated with apical volume oscillations and the hydrodynamic cycle (Fig. 2). Experimental data in pollen tubes and other plant cells show that cell hydrodynamics can activate and deactivate many key structural and signalling elements, including membrane trafficking, exocytosis and endocytosis, cell elongation and growth, ion flux, phospholipid and inositol polyphosphate signalling, and deposition and assembly of the apical pectin cell wall. Cell hydrodynamics has the potential to regulate other key elements including actin cytoskeletal organization and small GTPases. Future work will be directed to experimental investigation of these potential links.

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