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Mechanical Principles Governing Pollen Tube Growth

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ABSTRACT

Cellular growth and morphogenesis are central aspects of cellular differentiation. In plants, cellular growth is based on the turgor driven expansion of the cell wall and concomitant addition of new cell wall material. In no plant cell does this process occur as rapidly as in the pollen tube, the carrier of the male gametes. This cell is therefore an excellent model system to investigate the processes governing the dynamics of plant cell growth. This review provides a brief overview of the anatomy of the pollen tube focusing on the structural features that are implicated in the growth process – the cell wall and the cytoskeleton as well as spatially focused exocytotic events. The mechanics of the growth process is discussed and various theoretical modeling approaches that explain this process are outlined. In pollen tubes from many plant species the growth process is oscillatory or pulsatory and the ions and signaling molecules that form controlling feedback loops in this growth process are analyzed. A model that explains the oscillatory mechanism based on its mechanical components and that results from converging available data and hypotheses is elaborated.

Keywords: cell mechanics, cellular growth, cell wall, cytoskeleton, feedback mechanism, ions, modeling, oscillation, pollen tube, tip growth, turgor

Abbreviations: **ATP**, adenosine triphosphate; **ABP**, actin binding protein; **ADF**, actin depolymerizing factor; **ER**, endoplasmic reticulum; **GFP**, green fluorescent protein; **GTP**, guanosine triphosphate; **NAD**, nicotinamide adenine dinucleotide; **PME**, pectin methylesterase; **PtdInsP$_2$**, phosphatidylinositol 4,5-bisphosphate; **TIRF**, total internal reflection fluorescence

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INTRODUCTION

The pollen tube is formed upon contact of a pollen grain with a receptive stigma. The grain swells through water uptake and forms a cellular protrusion that invades the pistil. The purpose of this process is the transport of the male gametes from their carrier vehicle – the pollen grain – to the ovule where fertilization takes place. Depending on the species, pollen tube growth can occur extremely rapidly with rates up to tens and even hundreds of micrometers per minute. Very conveniently for the researcher, pollen grains are able to form these tubular protrusions *in vitro*, albeit usually not with the impressive growth rates observed *in planta*. However, cellular morphology of the former resembles that of the latter, which is why this single cell is an extraordinary system to investigate processes involved in

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plant cell growth.

Most types of plant cells grow by expanding large surface areas simultaneously. In the cylindrical cells composing stem tissues, this expansion occurs over the entire cylindrical surface, whereas only the end walls remain almost unaltered in size. Other cell types such as pavement cells in the leaf epidermis grow at more spatially confined sites to generate the typical jigsaw puzzle shape (Mathur 2006). Pollen tubes represent an extreme example of spatially confined growth since cellular expansion is limited to a single very small area at the apex of the growing cell. This mode of growth makes sense from an energetic point of view, because pollen tubes need to invade the transmitting tissue of the receptive flower. If the cylindrical wall of the tube expanded during growth, friction would occur against the surrounding tissue. Confining expansion to the apex minimizes the surface area on which friction occurs. This growth strategy is shared by several other cell types, which incidentally also have an invasive way of life. Among them are root hairs and fungal hyphae, and certain parallels can also be drawn to neuronal growth cones (Palanivelu and Preuss 2000). This illustrates that the tip growth strategy is realized in evolutionary very distant organisms.

Since pollen tubes are easily cultivated *in vitro*, and since even under this suboptimal condition their growth rate is impressive, this cell type has become an important model system for the investigation of the processes that govern plant cell growth. Numerous labs investigate different aspects of pollen tube growth which have been reviewed in countless articles and books (the most recent collection of articles can be found in Malhó (2006)). In the present review we will give an overview of the understanding of the mechanics and dynamics of the growth process. We will briefly present the structural features of the pollen tube and how their spatial distribution accounts for the highly anisotropic mechanism of growth. We will discuss the molecular feedback mechanisms that govern growth focusing on a particularity of the dynamics of the growth process – the oscillating change of the growth rate.

POLARITY IS REFLECTED IN THE CYTOARCHITECTURE

Cytoplasm

The anatomy of the pollen tube reflects the extreme polarization of the cellular processes. Starting from the apex, which is also called the growth zone, several distinct regions can be distinguished in the cytoplasm (**Fig. 1**). The apical dome is filled densely with secretory vesicles. The only other organelles that can occasionally be observed here are mitochondria and cisternae of the endoplasmic reticulum. The adjacent subapical and distal regions are densely populated by cellular organelles which move in a direction parallel to the longitudinal axis of the cell – towards or from the apex. This movement is rather rapid with rates of several micrometers per second. Most of these organelles turn around once they reach the subapical region (**Fig. 1A**). In larger pollen tubes such as lily, this rearward movement occurs mostly in the central area of the cytoplasm, and the flux is therefore characterized as "inverse fountain streaming" (Hepler *et al*. 2001). The shank of the tube contains the male germ unit consisting of the vegetative nucleus and the generative cell or, after its division, the two sperm cells. In longer pollen tubes, the viable part of the cytoplasm is concentrated in the region close to the apex and it is separated from degenerating distal regions and the pollen grain by callosic plugs.

This non-uniform distribution of cellular organelles is indicative of the compartmentalization of cellular functions (Cheung and Wu 2007), in particular with the growth zone being visibly different from the other regions of the cell. The polarity of the cell is also expressed in the non-uniform distribution of the concentrations of numerous molecules and ions and of the activities of certain enzymes. Among the principal players is calcium, which is highly concentrated in the apex and lower in the shank (**Fig. 1C**). Equally important are protons whose uneven distribution results in a pH profile that is characterized by a subapical alkaline band (Holdaway-Clarke and Hepler 2003; **Fig. 1D**).

Cytoskeleton

The pollen tube cytoskeleton is mainly composed of actin filaments and microtubules. Both are oriented in approximately longitudinal (or slightly helical) direction. Actin is found in the cortical cytoplasm as well as the endoplasm (Geitmann and Emons 2000). The shank of the tube in most species is characterized by the presence of numerous conspicuous actin bundles. On the other hand, the precise configuration of actin in the subapical region and in the apex varies between species as well as with the method of visualization. Important information has been gained from improved chemical or rapid freeze fixation combined with phalloidin staining, but dynamic aspects could only be studied using transformation with GFP (green fluorescent protein; or its derivatives) coupled to actin binding proteins (Kost *et al*. 1998; Chen *et al*. 2002). GFP coupled directly to actin has not been successful for pollen so far. The consensus of these studies is that angiosperm pollen tubes possess a subapical domain in which actin filament bundles are thinner and denser (also called actin fringe), whereas no significant amounts of filamentous actin are present in the very tip of the tube (**Fig. 1E**). The resolution of fluorescence images does not allow the precise characterization of the actin configuration in the fringe, not even when fixation is optimized (Lovy-Wheeler *et al*. 2005). A possible interpretation of the images could be that the front of the fringe corresponds to the advancing polymerization front, whereas the rear end represents the advancing actin bundling front (**Fig. 1E**). Alternatively, the fringe could represent a region of high degree of branching and the rear end might be a region of increased depolymerization. These hypotheses are not mutually exclusive. Depending on the degree of bundling either by the pollen tube's own protein machinery or by agents used to fix and stabilize actin, this fringe could thus appear longer or even funnel shaped as has been observed in several species such as maize, poppy and lily (Gibbon *et al*. 1999; Geitmann *et al*. 2000; Foissner *et al*. 2002). It will be interesting to see whether the corresponding actin binding proteins can be localized specifically to these "front" regions within the fringe that would correspond to high polymerization, depolymerization and/or bundling activities.

Several actin binding proteins (ABPs) act in the pollen cytoplasm to regulate actin polymerization and depolymerization. Villins have been proposed to degrade actin filaments in the apical region where the cytoplasmic Ca^{2+} concentration is higher thus resulting in a tip region that is relatively free of filamentous actin. In the distal region, where the Ca^{2+} concentration is at its basal levels, villin may act as a cross-linker, thus stabilizing the actin network (Kost *et al*. 1998; Yokota *et al*. 1998; Vidali *et al*. 1999; Yokota *et al*. 2000; Vidali *et al*. 2001). Profilin has also been shown to bind G-actin in the apical region, thus preventing actin polymerization (Kovar *et al*. 2000). Villin and profilin have a role in regulating the polymerization of actin filament bundles in root hairs (Baluška *et al*. 2000).

Actin depolymerizing factor (ADF) and cofilin are two ABPs that have the ability to segment and to depolymerize actin filaments. The segmentation activity of the ADF and the depolymerization activity of cofilin are both regulated by pH thus pointing to an important regulatory function of the pH profile (Lovy-Wheeler *et al*. 2006). Other ABPs were described in pollen tubes such as α-actinin (actin filament/cell wall link), formins (polymerization of actin filaments) and myosins. Several recent review articles on pollen tube actin and ABPs are available (Vantard and Blanchoin 2002; Staiger and Blanchoin 2006; Yokota and Shimmen 2006; Ren and Xiang 2007).

Microtubules are longitudinally oriented and sometimes

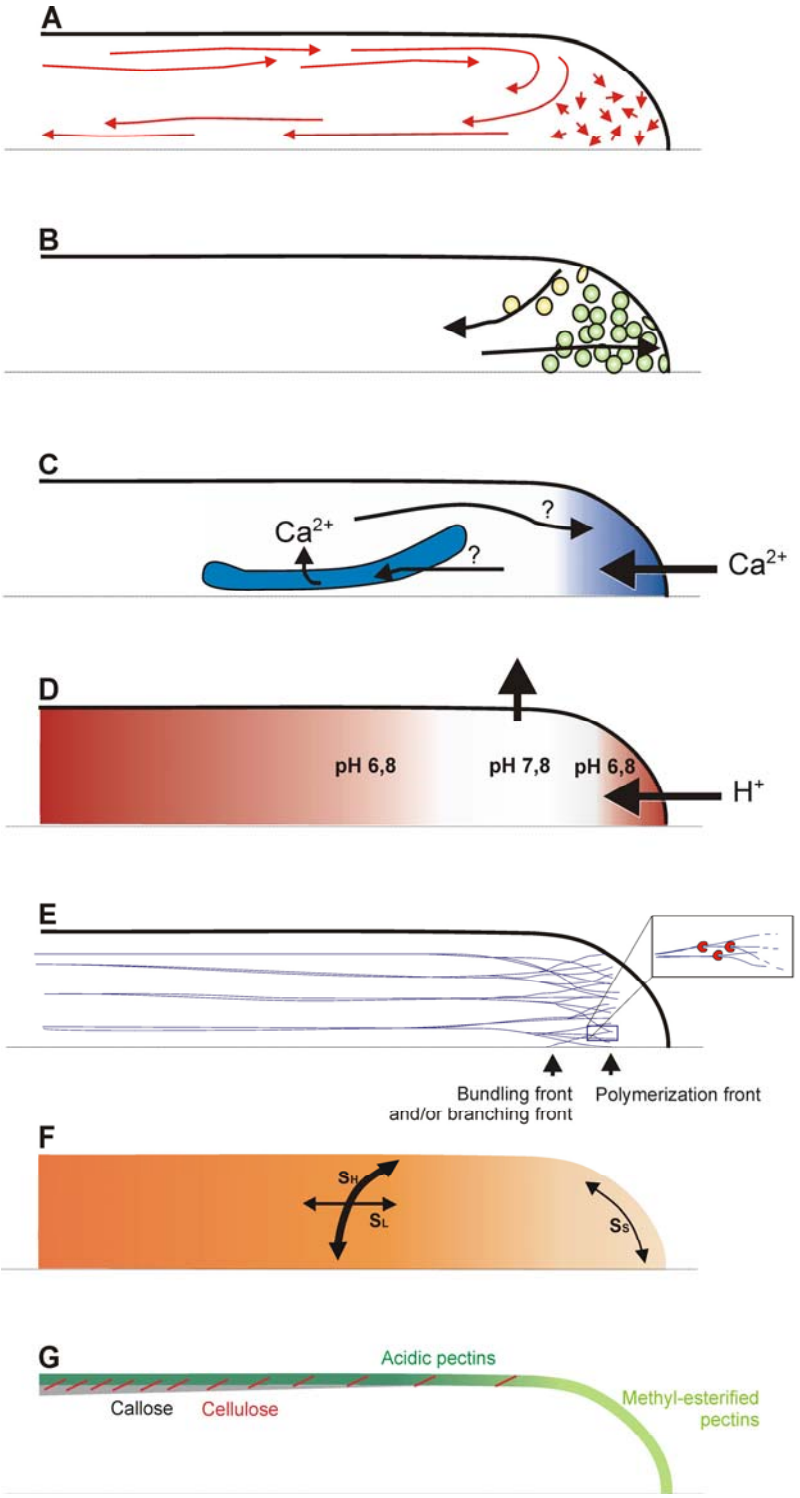

Fig. 1 Schematic representation of the polar arrangement of cellular structures and processes in the apical and subapical regions of a growing pollen tube. Because of the radial symmetry of the cell only half of a median section is drawn. Objects are not to scale. (**A**) Cytoplasmic streaming occurs parallel to the long axis. In larger tubes forward movement takes place in the cortex and rearward movement in the center of the tube. Vesicle movements in the apex are more erratic. (**B**) Secretory vesicles (green) accumulate in the apical region were exocytosis takes place. Rapid endocytosis likely takes place in the entire apex, but clathrin-mediated endocytosis (yellow vesicles) occurs predominantly at the base of the apex. (**C**) The cytosolic calcium concentration is high at the very apex and drops drastically towards the shank. Calcium influx occurs through the apical plasma membrane. It is unclear which organelles sequester calcium and to what degree calcium release from organelles contributes to the calcium gradient. (**D**) Proton influx occurs at the tip and efflux in the subapical region. This leads to the formation of an alkaline band that corresponds approximately to the turnaround point of cytoplasmic streaming and to the actin fringe. (**E**) Filamentous actin is arranged predominantly in longitudinal direction. Long cables characterize the shank region, whereas in the subapex actin seems to be branched or less bundled giving rise to a fringe. (**F**) According to the laws of thin-walled pressure vessels, the tensile stress in the wall generated by the internal pressure is twice as high in circumferential direction (S_H) compared to longitudinal direction (S_L) or the approximately hemisphere shaped tip (S_S). Shade of orange indicates the degree of cellular stiffness. (**G**) The biochemical composition of the cell wall changes from the apex to the distal region through decreasing methyl-esterification of pectins and addition of callose and cellulose.

adopt a slight helical distribution (Geitmann and Emons 2000). They extend from the pollen grain to the subapical region. In contrast to root hairs, microtubules are absent from the apical domain of angiosperm pollen tubes (Lancelle and Hepler 1992). The role of microtubules in polarized growth may be the control of directionality, because taxol (stabilizing agent) and oryzalin (destabilizing drug) have similar effects on the directionality of tube growth (Hepler *et al*. 2001; Gossot and Geitmann 2007). Recent reviews that summarize the role of microtubules in pollen tube tip growth include Geitmann and Emons (2000), Raudaskoski *et al*. (2001), and Cai *et al*. (2005).

Cell wall

The pollen tube cell wall is mainly composed of polysaccharides. The main component is pectin which is deposited at the apex by exocytosis. Cellulose and callose are laid down in more distal regions by plasma membrane localized synthases. In the case of *Nicotiana tabacum* cellulose deposits start at 5 to 15 µm from the tip whereas callose is visible starting at a distance of approximately 30 µm from the tip (Ferguson *et al*. 1998). The pattern of callose deposition is very universal, whereas there are considerable species dependent differences for cellulose localization. In *Arabidopsis thaliana* calcofluor label has indicated the presence of microfibrils in the apex (Derksen *et al*. 2002) whereas the apex of other species is devoid of cellulose.

Because of the general absence of a conspicuous "secondary" deposition of cell wall components in the apex, the overall biochemical structure of the cell wall differs considerably between the growing apex and the cylindrical shank of the cell (**Fig. 1G**). This difference is even more pronounced due to the gradual change in the configuration of

the pectins as they become part of the mature region of the shank. Labeling with monoclonal antibodies JIM5 and JIM7 has revealed that in growing angiosperm pollen tubes pectins have a higher degree of methyl-esterification in the tip than in distal regions (Li *et al*. 1994). For reviews on pollen tube cell wall structure see Heslop-Harrison (1987), Taylor and Hepler (1997), and Geitmann and Steer (2006).

MECHANICS OF ANISOTROPIC GROWTH

In general, the process of plant cell growth is driven by the relationship between turgor pressure, controlled water uptake and mechanical cell wall resistance. The controlled yielding of the existing cell wall under the applied pressure leads to an expansion of the cellular surface while simultaneously new cell wall material is inserted. The combination of these two processes results in a change of cellular shape. The situation is similar in a tip growing cell, with the particularity that the cellular expansion is confined to an extremely small region of the cellular surface. Similarly to diffuse growth, turgor is believed to be the primary motive force behind tip growth, albeit it might not be the rate-controlling parameter as growth rates cannot directly be correlated with the amount of turgor present in *Lilium longiflorum* Thunb (Benkert *et al*. 1997).

Given that hydrostatic pressure is a non-vectorial force, the question arises, how it can push a cell to produce a tubular protuberance instead of becoming a ballooning sphere. Green (Green 1969) and others have proposed that tip-localized expansion must be caused by tip-to-base changes in the physical properties of the wall. In other words, the cell wall in the shank of the tube needs to be more resistant to tensile stress than the apical cell wall to assure that the latter yields first thus allowing for tip-confined expansion. However, for geometrical reasons this difference actually has to be bigger than a factor 2. According to the physical laws for thin-walled pressure vessels, tensile wall stress in circumferential direction is twice as high as the tensile stress in longitudinal direction or that in the hemisphere-shaped ends of a cylindrical vessel (Geitmann and Steer 2006; **Fig. 1F**). The generation of a tubular structure therefore requires a considerable difference in the yield threshold between the shank and the apex. This difference in the physical properties of the pollen tube cell wall has been demonstrated by micro-indentation for *Papaver rhoeas* (Geitmann and Parre 2004) and other species.

Several cell wall components are likely to be responsible for this mechanical gradient in the pollen tube cell wall due to their non-uniform distribution (Heslop-Harrison 1987; Ferguson *et al*. 1998; **Fig. 1G**). Since both callose and cellulose are present predominantly in the shank of the tube they are presumed to play a reinforcing role in this part of the cell. Micro-indentation data as well as enzymatic and pharmacological approaches confirm this (Anderson *et al*. 2002; Parre and Geitmann 2005a). An even more important role is played by pectins and the spatial distribution of the degree of their methyl-esterification. Concerning the physical properties of the cell wall this is an essential feature as de-esterification allows pectins to become gelated in the presence of calcium ions. *In vitro*, this gelation process considerably increases the Young's modulus of this matrix component (Jarvis 1984), thus potentially increasing resistance against tensile stress. Micro-indentation revealed that this is also true for *in vivo* pollen tube cell walls (Parre and Geitmann 2005b). Furthermore, the enzymatic de-esterification and hardening of the usually rather soft apical wall is able to prevent pollen tube from elongating (Bosch *et al*. 2005; Parre and Geitmann 2005a), thus confirming the crucial role of the physical gradient in the mechanical properties of the cell wall for sustained growth.

THEORETICAL MODELS FOR UNIDIRECTIONAL GROWTH

The unidirectional growth typical for pollen tubes and other tip growing cells has inspired many attempts to model this process. Most of these were applied to fungal hyphae (Bartnicki-Garcia 2002), but the similarity between the cell types is such that the models could readily be transferred from one to the other. The early theoretical models for tip growth are based on equations that approximate the shape of these cells very closely. However, most of them are limited to geometric exercises that formulate equations from artificial coordinates and reference points (Reinhardt 1892; da Riva Ricci and Kendrick 1972; Trinci and Saunders 1977; Prosser and Trinci 1979; Koch 1982, 1994; Prosser 1994; Denet 1996). Few tried to take into consideration the geometrical and physical parameters of subcellular structures such as the thickness and chemical composition of the cell wall, the precise location of vesicle fusion or the hydrostatic turgor pressure. The vesicle supply center model for fungal hyphae by Bartnicki-Garcia *et al*. (1989) was an attempt to develop a mathematical model based on the intracellular path and secretion of secretory vesicles. The original two-dimensional mathematical formulation was based on the concept that tip growth is produced by wall-building vesicles emanating randomly, and in all directions, from a vesicle supply center which advances moving along a straight path. The subsequent three-dimensional derivation of this model included quantitative measurements of the pattern of expansion of the wall (Bartnicki-Garcia *et al*. 2000; Gierz and Bartnicki-Garcia 2001). Observations of surface expansion are also the basis of models developed by Dumais and co-workers for *Medicago truncatula* root hairs and further versions account for growth fluctuations and changes in morphology (Dumais *et al*. 2004, 2006). This was achieved by rescaling a given wall extensibility profile over time. The same group found interesting parallels between the tip expansion of lily pollen tubes and rubber balloons swelling under increasing internal pressure (Bernal *et al.* 2007). While the model by Dumais *et al.* considers the continuous supply of cell wall material to be provided in bulk form at the very tip of the cell, the vesicle supply center model was further developed by exchanging the theoretical ballistic path of secretory vesicles to the apical membrane for a more realistic diffusive vesicle delivery mechanism (Tindemans *et al*. 2006).

While being able to explain the formation of a cylindrical tube, many of these models nevertheless fail to include parameters important for the functioning of the living cell such as the turgor pressure and the non-uniform biochemical composition of the cell wall. An internal hydrostatic pressure was included in the approach by Goriely and Tabor who modeled the cell wall of a tip growing cell as a stretchable and growing elastic membrane with geometry-dependent elastic properties. They used large-deformation elasticity theory and combined the elastic response with surface re-parameterization to simulate wall rebuilding (Goriely and Tabor 2003a, 2003b).

Few of these models are directly based on geometrical and physiological data obtained specifically from pollen tubes. To allow for experimental validation, future modeling attempts will require precise quantitative data on the pollen tube geometrical features and the mechano-physical properties of its subcellular components. Cytomechanical approaches will therefore certainly gain popularity in this field (Geitmann 2006a, 2006b).

CONSTRUCTION OF THE APICAL CELL WALL: EXO-/ENDOCYTOSIS

To allow for the construction of the ever elongating tube, new cell wall material as well as membrane bound and secretory proteins need to be transported to the tip in a continuous manner and at a high rate. Exocytosis is therefore a *sine qua non* condition for pollen tube growth. In-

hibiting the vesicle supply by adding brefeldin A or monensin arrests pollen tube growth within a few minutes – presumably upon depletion of the apical stock of vesicles (Geitmann *et al*. 1996). The cell wall material deposited at the tip consists largely of pectin polymers which are transported to the apex within vesicles. This transport occurs via interactions between motor proteins linking the vesicle surface to the cytoskeleton. The actin cytoskeleton is thought to play a major role in this context and myosins have been identified in pollen tubes (Yokota and Shimmen 1994; Shimmen *et al*. 2000). However, dynein- and kinesin-like proteins have also been localized on pollen tube organelles indicating a transport function for microtubules as well (Moscatelli *et al*. 1998; Romagnoli *et al*. 2003, 2007). For reviews on motor proteins in pollen see Cai *et al*. (1996, 1997, 2005).

While the long-distance transport of vesicles along the cytoskeletal rail system in the tube shank is rather well understood, knowledge about the subsequent steps is scant. Once delivered to the apical region, vesicles seem to accumulate and somehow find their way to the apical membrane where they dock, fuse, and liberate their contents. Vesicle movement in the vesicle-rich growth zone was initially described as Brownian in *Nicotiana tabacum* (de Win *et al*. 1999). However, the situation is probably more complex since TIRF (total internal reflection fluorescence) microscopy has revealed that the movement in *Picea meyeri* pollen tubes is not random and seems to depend on a functional actin cytoskeleton despite the absence of conspicuous F-actin configurations in this area (Wang *et al*. 2006b). The apical actin cytoskeleton, possibly in form of dynamic individual F-actin arrays which are difficult to visualize, is therefore likely to be involved in guiding the vesicles to the designated fusion sites at the membrane.

Vesicle fusion must be spatially and temporally controlled. Spatial control serves to determine the direction of growth (Geitmann and Palanivelu 2007) and temporal control is likely to influence the dynamics of the growth rate. A crucial role in this control mechanism is said to be played by calcium (discussed below) which in turn acts via its activating or de-activating effect on a number of proteins. The signaling pathways targeting secretion are also thought to involve the inositide pathway and phosphorylation cascades (reviewed by Malhó *et al*. 2000, 2005, 2006; Geitmann and Palanivelu 2007).

Among the proteins that have been proposed to interact directly in vesicle secretion are annexins, SNAREs, and the members of the exocyst complex. Annexins are able to bind to, aggregate, and fuse secretory vesicle membranes in a calcium-dependent manner. They were immunolocalized in the vesicle-rich zone of *L. longiflorum* pollen tubes (Blackbourn *et al*. 1992) and are thus likely to be involved in the tip growth process. Annexin action is calcium dependent (Trotter *et al*. 1995) thus suggesting that the cytosolic high calcium concentration plays a role in directing vesicle fusion events.

The exocyst, first described in yeast (Novick *et al*. 1995) is composed of eight proteins functioning as a tethering complex guiding secretory vesicles to their specific plasma membrane site prior to the docking and fusion events mediated by SNARE proteins (Cole *et al*. 2005). In yeast, the exocyst was found to be associated with the plasma membrane at the site of cell-surface expansion (Novick *et al*. 1995). While orthologs of all exocyst components have been identified in *Arabidopsis* and rice, their role in tip growth has yet to be elucidated (Cole *et al*. 2005; Cole and Fowler 2006). However, mutants of SEC8, one of the exocyst components, were found to affect pollen tube growth or germination (Cole *et al*. 2005) and further investigations are likely to provide more insight.

SNAREs (soluble N-ethyl-maleimide-sensitive fusion protein attachment protein receptors) are integral membrane proteins. In plants, fusion of the vesicle with the target membrane depends on activated SNARE molecules in the vesicle (v-SNARE) and target (t-SNARE) membranes (Sanderfoot and Raikhel 1999; Nebenführ 2002). While SNAREs were found to be implicated in numerous plant functions (Pratelli *et al*. 2004), they have not been characterized or localized in pollen tubes in the context of exocytosis. Given the intensive secretory activity in these cells, their involvement is likely, however.

Two types of exocytosis have been proposed to occur in pollen tubes: full fusion and transient fusion. Using TIRF microscopy, Wang *et al*. (2006b) observed the full fusion type in *Picea* pollen tubes involving the collapse of the vesicles into the membrane as they release their internal components. Transient exocytosis and the resulting rapid endocytosis were proposed to occur in pollen tubes of various angiosperm species (Malhó *et al*. 2005). This exocytosis mechanism is a Ca^{2+}-dependent process coupled to endocytosis which requires GTP hydrolysis and dynamin but not clathrin (Monteiro *et al*. 2005). It is characterized by the formation of a small and short-lived pore which limits the size of particles that can be released or incorporated.

The observation of transient exocytosis points to the initially surprising finding that despite its rapid cell wall production rate and growth sustained by massive exocytosis, the pollen tube actually internalizes material at the tip at the same time. An explanation lies in the fact that an excess of membrane material is transported to the tip of the pollen tubes during exocytosis of cell wall material (Picton and Steer 1983b, 1985; Steer 1988; Derksen *et al*. 1995). Therefore, endocytosis of membrane is a necessity to maintain the relatively smooth outline of the apical plasma membrane. Next to rapid endocytosis, clathrin-mediated endocytosis is also purported to occur based on the presence of coated pits and vesicles carrying a clathrin-like coat in the tip area of tobacco pollen tubes (Derksen *et al*. 1995). The highest concentration of coated pits was observed in a region 6-15 µm behind the tip in *N. tabacum* pollen tubes (**Fig. 1B**). The bulk of endocytosis is therefore purported to occur not at the very apex, but slightly towards the shank. Both, ultrastructural research and, more recently, live cell observations that use fluorescent dyes which are internalized by endocytosis have contributed important information on the dynamics and spatial distribution of these events (reviewed in Malhó *et al*. 2005).

MECHANICS OF OSCILLATORY GROWTH

In addition to their rapid elongation, pollen tubes exhibit a dynamic feature which makes the investigation of their growth process extremely attractive: their growth rates fluctuate in regular intervals. This allows the investigation of the feedback mechanisms governing growth as signaling steps and physiological processes can be presumed to show temporal variations in intensity. Cross-correlation analysis of the phase shifts between these events and the growth rate should theoretically allow the identification of cause-effect relationships. A compilation of the parameters that have been assessed in this context is provided in **Fig. 2**.

While most pollen tubes show a non-continuous growth rate, the frequency and amplitude as well as the shape of the curve representing the growth rate varies considerably between species and depends on experimental conditions. Roughly, one can distinguish between oscillatory growth characterized by a sinusoidal curve and pulsatory growth in which short growth spurts are separated by extended periods of slow growth. In *L. longiflorum* the oscillations are approximately sinusoidal with a frequency of 15-50 sec (Pierson *et al*. 1996). In *N. tabacum* and *Petunia hybrida* pollen tubes show slow growth phases lasting few minutes (typically 3 to 8) interrupted by pulse-like elongations lasting few seconds. Pollen tube elongation during these pulses can reach up to 2 µm in *N. tabacum* achieving a peak growth rate up to 0.5 $\mu m.sec^{-1}$ (Geitmann 1997).

The species that is investigated in most detail is *L. longiflorum*. The most prominent oscillations are found in

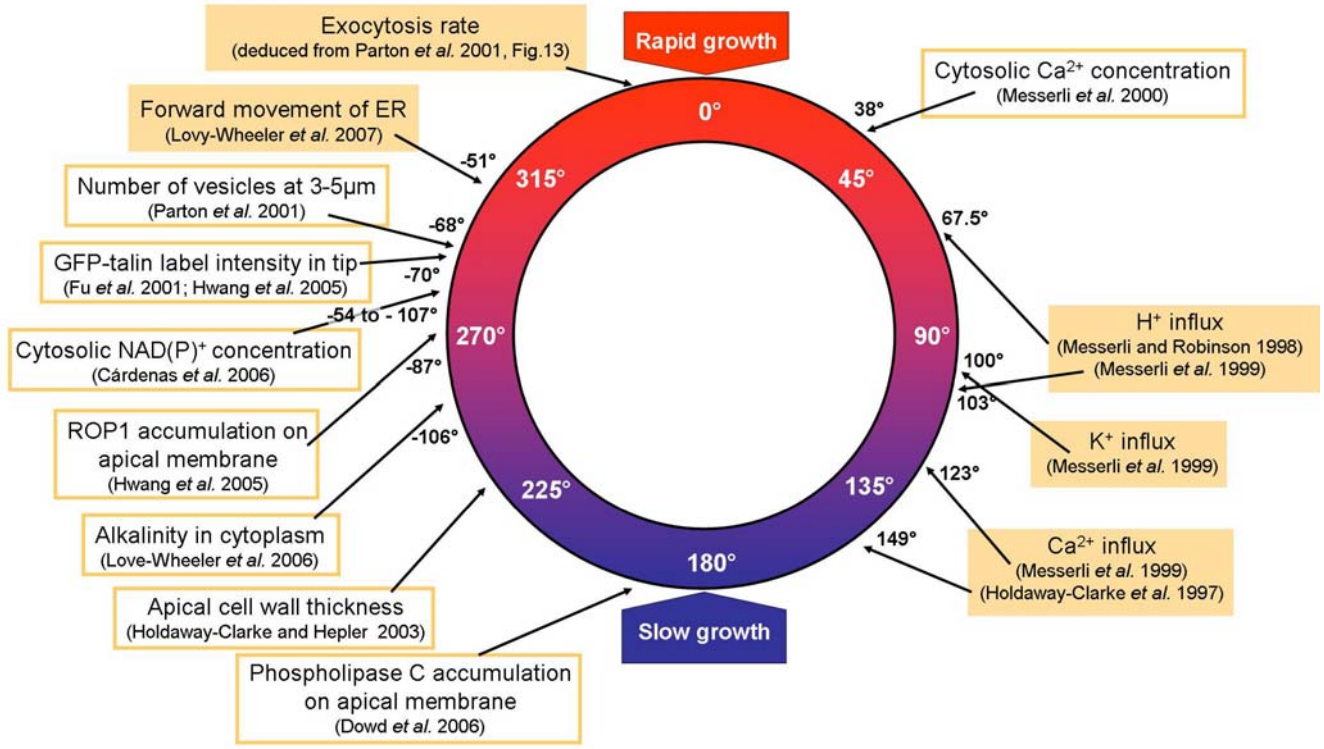

Fig. 2 Compilation of cellular features and processes that have been observed to undergo changes during oscillatory pollen tube growth. Note that these data have been acquired on pollen tubes from different plant species. Arrows indicate the phase relationships between peaks in concentration or flux rate of a particular parameter and peaks in growth rate. Phase shifts are indicated as degrees with 360° corresponding to a complete oscillation period. In most (but not all) cases this phase relationship has been established by cross-correlation analysis - a process that identifies whether a particular process is leading (negative numbers) or lagging (positive numbers) growth. Two classes of parameters can be distinguished: concentrations (orange frame) and movement rates (orange background). The latter are first derivatives of the former. This distinction is important when identifying cause-effect relationships. This becomes evident for example when looking at the label intensity for secretory vesicles, which is highest at -68°. For this particular parameter, however, more important than the *concentration* (the amount of vesicles), is the information that can be derived from the *change* of label intensity, since it corresponds to exocytosis activity, which in turn is a *rate*.

older tubes (>1 mm length). Lily pollen tubes are relatively easy to manipulate and to observe due to their considerable size (16-20 µm diameter vs. 6-10 µm in many other species). In the *in vitro* setup typical oscillations in this species have a period of 30-60 sec, and a growth rate oscillating between 0.1 and 0.4 µm.sec^{-1} (Messerli *et al*. 2004).

Despite the unidirectional manner of the pollen tube growth, these growth oscillations represent an ideal system to investigate the temporal relationship between mechanical events governing plant cell growth in general. The mechanical principle behind this oscillatory behavior is based on the changing relationship between the turgor pressure and the apical cell wall over short periods of time. The question is, which of the two – turgor or cell wall mechanics – is the mechanical oscillator in this system?

From the mechanical point of view, two models have been proposed: i) fluctuations in the mechanical properties of the cell wall allowing its relaxation under an approximately stable turgor, and ii) surges in hydrostatic pressure driving cell wall expansion. The former is based on the widely accepted understanding of plant cell growth as reviewed by Schopfer (2006). The latter is based on the "Loss-of-stability" principle proposed by Wei and Lintilhac (2006).

i) Fluctuations in cell wall physical properties

This model is based on the observation that the hydrostatic turgor pressure in growing pollen tubes does not seem to oscillate in *L. longiflorum* (Benkert *et al*. 1997). This finding is corroborated by the observation that two growing ends emanating from a single branched cell do not show the same oscillation frequency in *Petunia hybrida* (Geitmann 1997). If turgor was the determining oscillator, these branched pollen tube ends should oscillate with the same frequency, since the cell can be considered as a single volume in which turgor pressure can be assumed to be identical everywhere. It has therefore been suggested that the tensile strength of the apical cell wall and not the internal pressure varies during the oscillation cycles. This alternation between softening and hardening might for example be caused by the secretion of new cell wall material with high plasticity which allows rapid expansion and subsequently hardens either due to strain hardening or enzymatic activity (Geitmann 1999). It is interesting in this context to note that the thickness of the cell wall changes during an oscillation cycle. The peak of thickness precedes the most rapid phase of the growth pulse (Holdaway-Clarke and Hepler 2003). This phase relationship is intuitively necessary, since it assures the presence of sufficient cell wall material to allow for the subsequent expansion of the cell wall without the resulting thinning that would otherwise lead to rupture. On the other hand it is surprising, as this would mean that despite being thicker, the tensile stress resistance in the wall would actually have to be lower to allow for rapid expansion to occur. This would require that the newly added material is extremely soft.

Alternatively, the mechanical properties of the apical cell wall material could be controlled by the oscillatory activity of enzymes. The configuration of pectins, the most abundant polymers at the tube tip, is affected by various enzymes. Pectin methyl esterase (PME) acts on the methyl-esterified pectins that are secreted at the growing apex (Bosch *et al*. 2005). The degree of esterification is essential for cell wall mechanics as unesterified pectins are able to bind calcium ions resulting in the gelation of the polymers. This process rigidifies the wall as revealed by micro-indentation (Parre and Geitmann 2005b). It has been proposed that PME activity at the pollen tube tip is subject to a negative feedback mechanism (Holdaway-Clarke *et al*. 1997;

Bosch *et al*. 2005). According to this model a local decrease in pH generated by protons released during the deesterification reduces PME activity. This decrease in pH could also activate enzymes such as polygalacturonases and pectate lyases which affect the cell wall. The combined effect of the activation of these two enzymes and PME inactivation would loosen the cell wall and facilitate a growth pulse. The subsequent dilution of negative charges would then cause the pH to rise and thus reactivate PME and inactivate polygalacturonase and pectate lyase. This would lead to a slowdown of the expansion rate.

To test this hypothesis Messerli and Robinson (2003) experimentally varied the external pH or buffered the medium. They showed that a decrease in medium pH dampens growth oscillations while not increasing the average growth rate in *L. longiflorum*. The authors interpret the absence of a change in average growth rate as indicative for a turgor controlled growth rather than a cell wall control mechanism. However, we would argue that whereas changes of oscillation frequency and amplitude can be expected upon cell wall softening, the average growth rate over long intervals is likely to be limited by the rate of delivery of cell wall precursor material rather than by the deformability of the wall. Interestingly, buffering the external medium and thus presumably the cell wall pH, resulted in a reduction in growth frequency and an increase in pulse amplitude thus indicating that changes in the cell wall pH are probably not necessary for oscillating growth (Messerli and Robinson 2003). This finding calls the PME-pH model into question even though no proof was provided that the pH buffer in the medium effectively buffers the pH within the cell wall.

An alternative model does not rely on changes in the relative material properties of the apical cell wall, but takes into consideration the total tensile resistance determined by apical cell wall thickness. In this model a growth pulse is proposed to be triggered by a short phase of endocytotic uptake of cell wall material. This leads to a small reduction in cell wall thickness and thus increased deformability which in turn sets off rapid expansion under a turgor that remains relatively constant. This mechanism is suggested by a theoretical mathematical model for pollen tube growth oscillations (Kroeger, Grant, Geitmann, unpublished). Internalization of pectic wall material has been observed in other cellular systems (Baluška *et al*. 2002) thus making this hypothesis worth investigating experimentally.

Whether the cell wall or the turgor pressure is the driving factor in the oscillations, the cell wall properties most certainly do influence growth behavior. Agents that change these properties affect the frequency and/or amplitude of the growth rate or are able to induce pulsations in steadily growing pollen tubes. Borate cross-links monomers of rhamnogalacturonan II, a component of pectin polymers, and thus is known to stiffen the cell wall. It is generally added to the growth medium as pollen tubes require this ion for successful germination and growth. Surprisingly, the partial removal of borate from the growth medium causes growth pulse frequency to decrease in individual pollen tubes of *P. hybrida* (Geitmann 1997). The presence of high concentrations of borate in the medium from the beginning of germination on the other hand results in an increased oscillation period that can be explained with the stiffening effect on the cell wall (Holdaway-Clarke *et al*. 2003).

Similarly, in pollen tubes of *N. tabacum* external addition of PME decreases the frequency, whereas addition of auxin (indole-3-acetic acid) results in an increase (Geitmann 1997). Both agents were occasionally observed to trigger the onset of pulsating growth in steadily growing tubes. PME is known to rigidify the cell wall (Parre and Geitmann 2005b), and auxin is a cell wall loosening agent. Therefore, these findings indicate that during steady growth the tensile stress caused by turgor pressure and elastic tension of the cell wall are basically in an unstable equilibrium permitting continuous expansion. Upon disturbance in one direction or the other the finely tuned feedback processes become amplified and temporally distinguishable thus giving rise to a pronounced oscillatory behavior.

ii) Turgor surges

A rhythmic fluctuation of turgor pressure has been suggested to underlie force measurements with a miniature strain gauge on hyphae of the oomycete *Achlya bisexualis* (Johns *et al*. 1999). A similar principle has been proposed for pollen tubes (Derksen 1996; Geitmann 1999). It implies that an increase in cytoplasmic osmolarity leads to a rise of turgor driving rapid expansion. Messerli *et al*. (2000) then incorporated new metabolic data and suggested that turgor pressure rises preceding a rapid growth phase until it overcomes the yield threshold of the apical cell wall. In this model the wall is thickened preceding rapid growth and the membrane is slack due to bulk exocytosis which had taken place earlier. The growth surge then stretches both cell wall and membrane. Support for this model is provided by the observation that during experimental changes that correspond to lowering the growth rate amplitude, the slow growth rate is inversely proportional to the preceding peak of growth (Messerli and Robinson 2003). This can be explained if a surge in growth leads to a corresponding drop in turgor that decreases the subsequent growth rate.

Zonia *et al*. (Zonia *et al*. 2006; Zonia and Munnik 2007) attempted to corroborate the claim that turgor surges precede growth surges by measuring the diameter in the apical and subapical region of the cell. They observed that the cellular diameter in *N. tabacum* is at its maximum right before a growth peak. They suggest that the increase in the volume in this part of the cell indicates surges of hydrostatic pressure resulting from cycles of vectorial hydrodynamic flow. While their hypothesis has its merits, it is unfortunately based on the erroneous interpretation of cytomechanical data. The authors cite Geitmann and Parre (2004) stating that the mechanical properties of the cell wall do not change over time in growing pollen tubes. A high resolution time course that would have allowed them to draw this conclusion was, however, not assessed in the cited paper. Therefore, Zonia and Munnik cannot conclude from their volume changes to fluctuations in the turgor pressure, as volume changes could just as easily be achieved by a temporal change in the physical properties and subsequent yielding of the apical cell wall.

Zonia and Munnik make an interesting point, however, when they mention that the hydrostatic pressure in a single cell is not necessarily identical in all cellular regions. They cite Charras *et al*. (2005) and Langridge and Kay (2006) who observed that over short periods of time local cellular regions can undergo pressure changes that are only gradually equilibrated over the entire cell. This is supposedly possible because of the dense packing of the cellular components which could retain pressure. However, these findings were made on wall-less cells in which the hydrostatic pressure is several orders of magnitudes smaller than in plant cells. At the much higher pressures typical for a growing pollen tube (0.2 to 0.4 MPa, Benkert *et al*. 1997) it is doubtful whether a local pressure gradient could be established or maintained over a significant period of time. However, if proven it would invalidate the argument of the branched pollen tube mentioned above. More importantly, they would explain why turgor oscillations were not found in oscillating pollen tubes (Benkert *et al*. 1997) and they would indeed make any attempt to monitor local hydrostatic pressure over time in pollen tubes a huge challenge. Pressure measurements with a turgor pressure probe imply the impalement of the cell with a rather big micropipet, an endeavor that seems impossible to conceive in the growing apex of the tube. An alternative strategy to measure cellular turgor pressure relies on the external deformation of the cell by micro-indentation (Geitmann *et al*. 2004), atomic force microscopy or ball tonometry (Lintilhac *et al*. 2000; Wang *et al*. 2006a). However, while localized measure-

ments at the growing apex are possible with these devices, the experimental results would be influenced by changing cell wall properties rendering conclusions on turgor behavior complex. The ideal solution would therefore be the use of a non-invasive intracellular pressure indicator with spatial resolution, which to our knowledge has not been developed so far.

Vesicle fusion

The oscillations in cell wall thickness raise the question of how exocytosis behaves during an oscillation cycle. Is it a continuous process that causes an accumulation of cell wall material at the apex during slow growth, or does the rate of vesicle fusion change over time? The latter is likely, given that the cytosolic calcium concentration undergoes temporal changes (elaborated below) which is known to influence exocytosis rates in other plant and animal cell types (Battey *et al*. 1999; Beutner *et al*. 2001). A first indication that vesicle movement might play a role in the oscillations was provided by the result of pollen tube treatment with brefeldin A and monensin, two drugs interfering with vesicle transport. They cause pulsatory growth in *N. tabacum* to become steady thus abandoning the alterations in growth rate (Geitmann *et al*. 1996). Parton *et al*. (2001) then observed that fluorescence intensity of labeled vesicles close to the pollen tube tip oscillates with the same frequency as the growth rate. Fluorescence intensity at 3 to 5 µm from the apex peaks about 5-10 seconds before the growth rate (corresponding to an average phase shift of approximately -68°) and declines during the fast phase of growth. If one calculated the rate in the change of fluorescence intensity it seems from the graph provided by the authors that peaks in this rate change would pretty much coincide with peaks in growth rate. This indicates that a high rate of vesicle secretion occurs during fast growth. However, no statistical analysis was done to establish which of the two events starts first – secretion or expansion.

Other data that corroborate the hypothesis that massive vesicle fusion precedes or coincides with an increase in growth rate can be derived from observations of the dynamics of other organelle populations. Lovy-Wheeler *et al*. (2007) observed that the ER localized in the subapical region of growing pollen tubes moves forward in the cortical region preceding the peak of the growth rate by 4 sec (or -51°), followed by a "folding in" that fills the funnel-shaped ER accumulation to form a "platform". This occurs by 3 sec (or -36°) before the growth peak [The discrepancy between the numbers in seconds and degrees arises from different sample sizes and average growth periods]. The authors purport that this ER movement is actin-myosin based and thus active. A different interpretation of the data is possible, however. If one assumes that growth peaks are preceded by massive exocytosis, the number of secretory vesicles and thus the cytoplasmic volume they occupy in the apex should be reduced considerably during such episodes. The resulting deficit in cytoplasmic contents has to be filled and ER lying adjacent to the tip could thus be "sucked" into the tip in a passive manner. In this scenario, the forward movement of the ER simply reflects the secretory activity thus in turn providing information on the temporal relationship between growth rate and exocytotic activity.

Interestingly, Parton *et al*. (2001) also observed a backward movement of vesicles during slow growth in *L. longiflorum* and speculated that this corresponds to a cycling of excess material that is not used during slow growth phases. Intriguingly, Parton *et al*. (2003) found later that pollen tubes whose growth had been halted through brefeldin A action still exhibit periodic vesicle cloud movements in the apical cytoplasm. However, being about five times higher these events have a frequency that is significantly different from that of the typical growth rate fluctuations. The authors propose that these movements nevertheless provide the underlying periodicity for growth rate fluctuations and that the reason for the difference in frequency is the lack of a feedback signal in non-growing tubes.

OSCILLATORY GROWTH - CONVERGING THE MODELS

Because of its relatively rapid oscillatory behavior pollen tube growth has become a model system *par excellence* for understanding the feedback mechanisms that govern plant cell growth. From the data summarized above it becomes clear that neither turgor pressure nor cell wall is the exclusive factor that generates oscillations, but that both influence growth behavior. Due to limitations of space, not all experimental data were summarized here, but we will nevertheless try to propose a converged model that is consistent with most of the observations that have been made. More importantly, this model considers the fact that both of the above mentioned models tacitly acknowledge that the "stable" component (cell wall or turgor) does in reality have to undergo some degree of fluctuation. For example, a turgor-driven expansion of the cell will cause the cell wall to thin and/or strain-harden during the process thus altering its physical properties. Alternatively, a growth surge set off by the softening of the cell wall will to some degree alter the hydrostatic pressure in the cell.

We do agree that the cell wall and the turgor pressure are the two mechanical components that determine cellular growth. We suggest that during steady growth, pollen tube expansion is defined by finely tuned feedback mechanisms that react quasi-instantaneously. This situation can be compared with a mechanical system in equilibrium, such as a mass attached to a spring, in which the weight of the mass is in equilibrium with the tension of the spring. The visible result in the pollen tube is a steady expansion rate. Upon disturbance, the system would enter an oscillatory rhythm in which the feedback mechanisms are still at work but are now spatially and temporally resolvable. This corresponds to the mechanical mass being displaced and the spring exerting a restoring force on it. This restoring force, however, does not succeed in stabilizing the mass at its original position, since the mass takes up momentum (kinetic energy) and passes this position. This is the principle of a harmonic oscillator. While the movement of the mass-spring setup will dampen after a while and the oscillations will decay as a result, this can be prevented by a continuous energy transfer from the environment. An example would be the phenomenon of flutter in aerodynamics.

The idea of comparing the pollen tube to an oscillator is by no means new (for an excellent analysis refer to Feijó *et al*. 2001). However, in the past many attempts were done to identify the one parameter that drives the oscillations - a signal generator. If we look closer at the mechanical laws of a simple harmonic oscillator, however, a signal generator is not needed. The only elements required are the following:

i) *An equilibrium between forces*

The tensile force on the cell wall generated by the presence of the turgor pressure, which is counteracted by the elastic tension determined by the deformability of the cell wall.

ii) *An initial disturbance*

A disturbance of the equilibrium could be achieved by a direct interference with turgor pressure (through changing the osmotic value of the medium) or with cell wall properties (through applying digestive enzymes or cross-linking agents). Alternatively, this effect can be generated indirectly by changing the cytoplasmic calcium concentration, calcium fluxes, proton concentrations etc. all of which result ultimately in a change of turgor or in an alteration of the deformability of the cell wall through addition of cell wall material and/or through its rigidification or softening.

iii) *A robust feedback mechanism*

This feedback mechanism that is likely to comprise many components (see below) amplifies the initial disturbance into a stable oscillation.

iv) *A transfer of energy*

This energy is supplied by the pollen tube metabolism. It prevents the two acting forces to balance out and thus sustains the oscillations. In the case of the pollen tube this energy is supplied in form of cell wall material that is delivered to and liberated at the apex.

A theoretical model that illustrates that these four parameters are sufficient to generate oscillatory behavior has been proposed by Kroeger, Grant, Geitmann, unpublished. We can therefore abandon the search for a signal generator. Neither the cell wall nor the turgor generates the oscillations, but both contribute to controlling the outcome by influencing frequency and amplitude.

The great advantage of this model consists in the fact that despite its simplicity, it explains most of the experimental data. All the cellular processes that have been studied in the context of oscillatory growth ultimately affect either cell wall, turgor pressure or energy supply. For example the effect of cytoskeletal inhibitors can be explained by the fact that they interfere with vesicle delivery. According to the model (point *iv*) sufficient energy needs to be supplied to sustain oscillations, otherwise they are dampened over time. This is indeed observed in the experimental situation as oscillations are attenuated upon the addition of cytochalasin D, an inhibitor of actin polymerization (Geitmann *et al*. 1996). The model is also consistent with the fact that lily pollen tubes typically start out with steady growth behavior and switch to oscillatory growth at a later stage. It also explains that both cell wall softening and rigidification can induce pulsatory growth in previously steadily growing pollen tubes.

ION-BASED PARAMETERS INFLUENCING OSCILLATORY GROWTH

To understand the components of the feedback mechanism influencing the growth oscillations in pollen tubes it is helpful to identify reactions, activities and molecular concentrations that oscillate with the same frequency as the growth rate, but not necessarily in the same phase. Cross-correlation analysis allows then to identify the temporal relationship between these parameters. The first group of molecules for which the temporal behavior was investigated were ions, such as Ca^{2+}, K^+, H^+ and Cl^- (for reviews see Feijó *et al*. 2001; Holdaway-Clarke and Hepler 2003; Hepler *et al*. 2006). Two approaches were used – the visualization of temporal changes in the local cytoplasmic ion concentrations using fluorescent dyes and the measurement of ion fluxes using a vibrating probe. The vibrating probe is an electrode filled with ion exchanger liquid that moves back and forth between two positions measuring the difference in electric potential between them (Jaffe and Nuccitelli 1974). From this information the flux of ions in the adjacent region (in this case between the cell and the surrounding medium) can be extrapolated.

Calcium ions

The presence of Ca^{2+} in the growth medium is a necessity for successful germination of most pollen species (Brewbaker and Kwack 1963; Picton and Steer 1983a). Cytosolic Ca^{2+} concentration has been measured in various species and using different dyes, such as the ratiometric indicator dyes indo-1 (Rathore *et al*. 1991), and fura-2-dextran (Pierson *et al*. 1994, 1996; for a comparison of methods see Camacho *et al*. 2000), or the Ca^{2+}-sensitive photoproteins aequorin (Messerli *et al*. 2000) and cameleon (Iwano *et al*. 2004; Watahiki *et al*. 2004). All the studies show that there is a striking tip-focused gradient in cytosolic Ca^{2+} concentration. Depending on species and method used the cytoplasmic region closest to the tip shows a Ca^{2+} concentration between 3 and 10 μM which drops to tens or hundreds of nM within 20 μm from the apex. This gradient is maintained by an influx of Ca^{2+} ions through the apical membrane (Feijó *et al*. 1995a, 1995b; Messerli *et al*. 1999) mediated by Ca^{2+} channels (Dutta and Robinson 2004). While the principal source of Ca^{2+} seems to be extracellular, Ca^{2+} release from internal stores, conceivably from vesicles or ER located in the apical region, is likely to contribute (Kost *et al*. 1999; Hepler *et al*. 2001). The rapid decrease in Ca^{2+} concentration in the subapical region might be due to ion uptake into intracellular stores such as mitochondria, or the binding of Ca^{2+} to secretory vesicles, but no experimental evidence exists so far.

The presence of the Ca^{2+} gradient is closely coupled to growth, since treatment with BAPTA-type buffers (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), which dissipate the gradient, also arrest growth (Rathore *et al*. 1991; Miller *et al*. 1992; Pierson *et al*. 1993). Inversely, growth arrest due to other factors is accompanied by a dissipation of the Ca^{2+} gradient (Franklin-Tong *et al*. 1997). The cytosolic Ca^{2+} is thought to be a key player in the intracellular signal transduction and integration. These signaling pathways have been shown to also be based on phosphoinositides, phospholipases and Rho GTPases (reviewed in Franklin-Tong 1999; Malhó *et al*. 2000, 2006; Geitmann and Palanivelu 2007). The high cytosolic Ca^{2+} concentration at the very apex has been postulated to provide the spatial information for the localization of exocytosis events. This is confirmed by the observation that the artificial displacement of the highest Ca^{2+} concentration through local photorelease of caged Ca^{2+} alters the growth direction of the tube (Malhó *et al*. 1994, 1995; Malhó and Trewavas 1996).

It was therefore not surprising that Ca^{2+} also seemed to temporally determine growth rate since its concentration oscillates at the same frequency as the growth oscillations (Holdaway-Clarke *et al*. 1997; Messerli and Robinson 1997). What was surprising, however, was that the peaks in Ca^{2+} concentration do not precede growth peaks, but are delayed by several seconds in pollen tubes of *L. longiflorum* (Messerli *et al*. 2000; Hepler *et al*. 2006). This corresponds to a phase lag of 38° (Messerli *et al*. 2000). The peak in the influx of Ca^{2+} into the apex is even more delayed; values for this phase lag vary between 123° (Messerli *et al*. 1999) and 149° (Holdaway-Clarke *et al*. 1997). This phase relationship indicates that Ca^{2+} is unlikely to be a determining factor in rapid growth events but rather a consequence. However, especially the phase relationship between calcium flux and growth might be skewed by the fact that the cell wall might act as a Ca^{2+} buffer. Anionic sites on newly secreted pectin polymers can bind Ca^{2+} ions and therefore the Ca^{2+} measured with the vibrating probe actually only reflects the Ca^{2+} flux from the external medium into the cell wall. This flux is likely not to correspond to the amount (Holdaway-Clarke and Hepler 2003) nor to the timing of the Ca^{2+} flux across the plasma membrane into the cytosol.

Another possible explanation for the temporal relationship between Ca^{2+} flux and rise of cytosolic Ca^{2+} concentration is the assumption that the main source of the latter lies in intracellular stores. Messerli *et al*. (2000) propose that Ca^{2+} influx through the membrane only raises the cytosolic Ca^{2+} concentration to some critical threshold which then triggers the massive release of Ca^{2+} from internal stores, which is in turn the signal observed in the fluorescence microscope. Evidence for this hypothesis is provided by the biphasic shape of the curve representing the Ca^{2+} surge (Messerli and Robinson 1997).

To understand the role of transmembrane Ca^{2+} flux for oscillating growth, manipulation of external Ca^{2+} concentration as well as pharmacological approaches to inhibit Ca^{2+} specific ion channels were used. The ten-fold increase of external calcium concentration decreased the amplitude of growth oscillations while increasing the basal growth rate in *L. longiflorum* (Messerli and Robinson 2003). The former can be explained by the cross-linking and thus stiffening effect of Ca^{2+} on the cell wall pectins, whereas the latter might be related to an increase in exocytosis rate due to an increase in cytoplasmic Ca^{2+} concentration. In-

creased calcium concentrations in the medium also increased the oscillation period (Holdaway-Clarke *et al*. 2003). However, drawing conclusions from the data is not straightforward, since externally applied Ca^{2+} can have at least two effects: modifying the physical properties of the cell wall and changing the intracellular calcium concentration leading to an effect on exocytosis. To eliminate the effect of Ca^{2+} on the cell wall from the equation, inhibitors of calcium channels have been applied to assess the effect of a reduction in Ca^{2+} influx on growth. In particular La^{3+} and Gd^{3+} successfully reduced pulsation frequencies in *N. tabacum* (Geitmann and Cresti 1998) indicating that the influx rate of calcium affects the oscillatory behavior. A recently developed theoretical model for the role of Ca^{2+} in oscillatory growth fits most of these experimental data very well (Kroeger, Grant, Geitmann, unpublished).

Protons

The medium pH is a very critical condition for *in vitro* pollen tube growth. In lily pollen tubes, the optimum pH for growth is situated between 5 and 6. When the extracellular pH reaches 7, it is unable to support lily pollen tube elongation. In *Arabidopsis* on the other hand, the optimal pH is around 7, lower values reduce the germination rate. Like Ca^{2+}, H^+ concentration in the extracellular medium is higher than its concentration inside the pollen tube. This imbalance in concentration and electric potentials will create a strong force that will cause these ions to enter the cell. Holdaway-Clarke and Hepler (2003) suggested that H^+ may enter at the tip of pollen tubes by using the same non-specific cation channel as Ca^{2+}.

Initially, no intracellular pH gradient was thought to be present in the pollen tube cytoplasm. This was due to limitations in the methods used (Fricker *et al*. 1997). The problems were based on the high mobility of H^+ ions (compared to Ca^{2+}), the predisposition of dyes to bleach rapidly and the fact that the typical indicator dyes, especially at elevated concentrations, dissipate these presumptive gradients (Holdaway-Clarke and Hepler 2003). Low concentrations of BCECF-dextran indicator were eventually used successfully to visualize a pH gradient in lily pollen tubes. The apex of these cells is characterized by a slightly acidic domain (pH = 6.8), whereas the base of the clear zone is alkaline (pH = 7.5) (Feijó *et al*. 1999). Using a vibrating probe, Feijó *et al*. (1999) showed also that there is a proton influx at the extreme apex of the *L. longiflorum* pollen tube and an efflux in the region corresponding to the alkaline zone (**Fig. 1D**). They also demonstrated that the alkaline band correlates with the position of the turnaround point of the reverse fountain streaming at the base of the clear zone. It is thought that this alkaline zone is governed by a plasma membrane H^+-ATPase (Hepler *et al*. 2006). It had been shown previously that the activity of the H^+-ATPase seems to regulate the growth rate of pollen tubes. Treatment with an ATPase antagonists such as vanadate inhibits tube growth in lily whereas agonists like fusicoccin, increase pollen tube growth rate (Fricker *et al*. 1997). When pollen tube growth is inhibited, the apical acidic band disappears while the alkaline zone extends.

H^+ influx at the tip oscillates with the same phase as the growth oscillations, but the peak lags behind by 67.5° (Messerli and Robinson 1998) or 103° (Messerli *et al*. 1999). Correspondingly, cytosolic pH becomes more acidic in the tip with changes up to a full unit during a growth cycle with the cytoplasmic acidification following a rapid growth peak (Messerli and Robinson 1998; Feijó *et al*. 1999). The authors propose that the rise in cytosolic pH following a growth pulse may lower the affinity of Ca^{2+} binding proteins for Ca^{2+} thus shutting off Ca^{2+}-triggered vesicle fusion. While cross-correlation analysis indicated that acidification follows growth, alkalinization was observed to actually precede growth peaks by in *L. longiflorum* and *Lilium formosanum* (Lovy-Wheeler *et al*. 2006). The alkaline band coincides spatially with the cortical actin fringe in these species suggesting that the changing pH might affect the actin cytoskeleton, for example via the action of ADF. Lovy-Wheeler *et al*. (2006) proposed that the ADF is stimulated by low pH to fragment F-actin in the cortical fringe resulting in the exposure of new plus or barbed ends that in turn enhance new polymerization of actin.

A change in external proton concentration does not affect pollen tube oscillations very dramatically within the range of pH that permits pollen tube elongation (Holdaway-Clarke *et al*. 2003). However, a lower pH in the external medium has the tendency to increase the amplitude characterizing the growth oscillations. In addition to affecting the cytoplasmic pH, protons are also cell wall loosening agents; in pollen tube walls they may act through the enzyme PME, and either reduce demethylation or stimulate hydrolysis of pectin.

Potential players: Chloride and potassium ions

Chloride ions have been shown to play a role in pollen tube growth. The inhibition of presumed chloride channels by inositol-3,4,5,6-tetra*kis*phosphate (Cl^- channel blocker used in animal cells) showed that the pollen tube growth rate diminished accompanied by a swelling of the cell (Zonia *et al*. 2002). Because of problems with the specificity of the vibrating probe to Cl^- channels, reliable data on Cl^- flux oscillations are not available yet (Messerli *et al*. 2004).

The existence of various types of K^+ channels in pollen tube plasma membrane and in pollen grains have been evidenced using patch-clamp techniques in *Brassica*, lily and *Arabidopsis* (Obermeyer and Kolb 1993; Fan *et al*. 1999, 2001; Griessner and Obermeyer 2003; Dutta and Robinson 2004). In *Arabidopsis*, a mutation in the K^+ channel reduces ion uptake in pollen tubes and consequently growth (Mouline *et al*. 2002). Messerli *et al*. (1999) measured tip-restricted K^+ fluxes in lily pollen tubes using the vibrating probe and observed a lag of 100° with respect to the growth rate. The pulses of K^+ and H^+ are very similar suggesting that K^+ is taken up via a K^+/H^+ co-transporter (Messerli *et al*. 1999). The authors propose that the K^+ influx restores either the total ionic concentration and/or osmotic concentration in the cytoplasm after the increase in cell volume resulting from the growth pulse. This would help the cell to rapidly recover turgor pressure for the increased volume of the tube.

OTHER OSCILLATING PARAMETERS

NAD(P)H

NAD(P)H are essential coenzymes with a central role in the control of cellular metabolism. Their high energy status and reducing power drive many key biosynthetic reactions and ATP production. Because NAD(P)H but not $NAD(P)^+$ possesses an endogenous fluorescence, the reduced form can be detected in living cells. Cárdenas *et al*. (2006) showed that the strongest signal for NAD(P)H in lily pollen tubes is observed 20-40 μm behind the apex, where mitochondria accumulate. This suggests that $NADP^+$ may be coupled to ATP synthesis in mitochondria. The cytosolic concentration of NAD(P)H is observed to change during oscillatory growth. Peaks of NAD(P)H follow growth peaks by 77° to 116° (7 to 11s) whereas troughs anticipate growth maxima by 5 to 10 sec corresponding to -54° to -107°. This oscillation was suggested to be due to a periodic change of state of NAD(P)H from reduced to oxidized form thus suggesting that growth peaks might be preceded by increases in $NAD(P)^+$ (Cárdenas *et al*. 2006). It is therefore possible that the transformation of NAD(P)H to $NAD(P)^+$ is coupled to ATP synthesis, which is then harvested to power energy-consuming processes located at the pollen tube apex. Among these could be the proton-pumping ATPase responsible for the alkaline band, actin polymerization (given that G-actin bound to ATP is the prefer-

able form of the monomer), and exo- and endocytosis.

Small GTPases and actin

Rho-family small GTPases are important signaling switches in all eukaryotes. A single subfamily has been identified in plants (ROPs: rho-like GTPase from plants). These proteins are known to coordinate various pathways regulating cellular activities such as the production, targeting and fusion of secretory vesicles, remodeling of cell wall, Ca^{2+} gradient and other signaling pathways (Hwang and Yang 2006). ROP1 is highly expressed in mature pollen grains and in its active form it forms a tip-high gradient in the extreme of the pollen tube plasma membrane. The tip-localized activity of ROP1 is dynamic as it oscillates with the same frequency as the growth rate (Hwang *et al*. 2005). The maximum of ROP1 accumulation precedes growth rate by 87°, thus supporting a crucial role of this protein in the control of pollen tube growth. It is not well understood how ROP regulates growth, but it is known that it acts on F-actin dynamics mediated by downstream effectors such as RIC3 and RIC4 (Gu *et al*. 2005). This points at the importance of visualizing filamentous actin in growing pollen tubes to characterize its dynamics in relationship to the different growth phases. Labeling with GFP-talin has provided first indications that the configuration of the actin cytoskeleton varies over time. Fluorescence intensity of the talin marker in the apex precedes growth rate by -70° (Fu *et al.* 2005; Hwang *et al*. 2005). However, given that talin is an ABP whose dynamics do not necessarily reflect the dynamics of the actin filaments, more detailed information is elusive at present. To obtain these data a labeling method for actin needs to be developed that allows the satisfactory visualization of the apical actin population in living pollen tubes without interfering with its functioning. To date, the available methods have not been able to combine both of these requirements (Wilsen *et al*. 2006).

Phospholipase C

The inositol signaling pathway relies on the activity of phospholipase C, an enzyme that cleaves phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) into two cellular regulators: diacyl glycerol and inositol 1,4,5-trisphosphate. Phospholipase C has been localized to the apical plasma membrane in pollen tubes of *Petunia inflata* (Dowd *et al*. 2006). Pollen tubes from this species do not show sinusoidal oscillations, but rather extended phases of slow growth interrupted by short growth pulses. It was observed that phospholipase C accumulates on the apical plasma membrane during slow growth whereas during a rapid growth phase the enzyme leaves the membrane and accumulates in the cytoplasm. The presence of the enzyme on the plasma membrane during slow growth results in low levels of $PtdInsP_2$, whereas these levels rise during rapid growth. Dowd *et al*. (2006) propose that this increase in $PtdInsP_2$ sustains growth through regulation of membrane dynamics and/or alterations in cytoskeletal structure. However, the precise phase relationship between accumulation of phospholipase C and growth activity was not assessed and causal relationships cannot be drawn directly from these data.

POLLEN TUBE GROWTH *IN PLANTA* - GUIDANCE AND INVASION

While pollen tubes are an excellent model system to be studied *in vitro*, there is a lot of interest in the *in vivo*, or *in planta* situation. The situation of a pollen tube growing *in planta* is a very particular situation, since it represents the invasion of one organism (the sporophyte that carries the female gametophyte and future fertilization partner) by another (the male gametophyte). The questions that arise concern in particular recognition mechanisms occurring during cross- and auto-incompatibility reactions (recently reviewed by Silva and Goring 2001; Takayama and Isogai 2004) and guidance mechanisms allowing the pollen tube to find its way within a compatible pistil (recently reviewed by Palanivelu and Preuss 2000; Cheung and Wu 2001; Geitmann and Palanivelu 2007). In the context of this review we will focus on the mechanical aspect of pollen tube growth through the female tissues of the pistil.

During the invasion of the stigmatic and stylar tissues, in particular in species with solid styles, growing pollen tubes have to invade the apoplast of the stylar transmitting tissue. Even though pollen tubes are able to soften these tissues either by inducing programmed cell death or by enzymatically digesting the cell walls (Hiscock *et al*. 1994; Greenberg 1996; Wang *et al*. 1996; Hiratsuka *et al*. 2002; Suen and Huang 2007), these processes are unlikely to completely liquefy the growth matrix. The advancing apex of the elongating pollen tube, therefore, has to be able to exert sufficient penetration force and to withstand externally applied compressive stress. While most of the growth force is thought to be provided by the hydrostatic turgor pressure in the cell it was observed that the actin cytoskeleton might be involved in generating an invasive force. Treatment with low concentrations of latrunculin B, an inhibitor of actin polymerization, does not inhibit pollen tube growth but reduces the pollen tube's ability to invade a stiffened growth matrix in the case of *Papaver rhoeas* (Gossot and Geitmann 2007). Whether the force generated by the polymerization of filamentous actin actually forms a mechanical contribution to the invasive force is questionable, however, as these forces have been calculated to be very small compared to that generated by the turgor pressure (Money 1997). However, it is interesting that actin polymerization inhibitors at low concentrations also inhibit growth oscillations in pollen tubes (Geitmann *et al*. 1996). It is, therefore, not altogether absurd to speculate that these growth oscillations might facilitate the invasive growth in pollen tubes (Geitmann 1999).

This idea is particularly intriguing if one considers that in many cases the shape of the pollen tube changes upon the initiation of a rapid pulse phase. The rapidly elongating apex has a smaller diameter that broadens during the subsequent slow phase (Geitmann 1997; Zonia *et al*. 2001). While intuition suggests that a smaller apex penetrates easier into a stiff matrix, the physical background can be found in fracture theory. Based on energy conservation principles (Callister 1994) it can be calculated that the smaller the width of a crack in a material, the bigger is the stress on the material that enables the propagation of the crack. This could explain why a pollen tube during rapid elongation would be more successful in penetrating the surrounding matrix if its apex was smaller.

CONCLUSIONS

In no other cell type can the dynamics of plant cell growth be observed in such a dramatic manner as in pollen tubes. Not only do these cells grow extremely rapidly, but also is the growth behavior oscillatory. This temporal separation of events allows for the investigation of feedback mechanisms that determine the growth process. While numerous pieces of the puzzle have been identified, the completion of the picture will require the acquisition of a lot of additional data and, more importantly, their analysis and interpretation. We proposed a simple mechanical model for oscillatory growth that renders the search for a signal generator superfluous. It is based on three mechanical elements: the turgor pressure, the cell wall deformability and a continuous supply of energy. All other parameters that are known and will be found to influence oscillatory growth ultimately act via their effects on one or several of these key elements. This model might help to understand numerous aspects of the pollen tube growth process and is amenable to refinement.

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