Polar growth in pollen tubes is associated with spatially confined dynamic changes in cell mechanical properties

Rabah Zerzour\textsuperscript{a}, Jens Kroeger\textsuperscript{b}, Anja Geitmann\textsuperscript{a}
\textsuperscript{a}Institut de recherche en biologie végétale, Département de sciences biologiques, Université de Montréal
4101 rue Sherbrooke est, Montréal, Québec H1X 2B2, Canada
\textsuperscript{b}Physics Department, McGill University, 3600 rue University, Montréal, Québec H3A 2T8, Canada
Corresponding author: anja.geitmann@umontreal.ca

Abstract

Cellular morphogenesis involves changes to cellular size and shape which in the case of walled cells implies the mechanical deformation of the extracellular matrix. So far, technical challenges have made quantitative mechanical measurements of this process at subcellular scale impossible. We used micro-indentation to investigate the dynamic changes in the cellular mechanical properties during the onset of spatially confined growth activities in plant cells. Pollen tubes are cellular protuberances that have a strictly unidirectional growth pattern. Micro-indentation of these cells revealed that the initial formation of a cylindrical protuberance is preceded by a local reduction in cellular stiffness. Similar cellular softening was observed before the onset of a rapid growth phase in cells with oscillating growth pattern. These findings provide the first quantitative cytomechanical data that confirm the important role of the mechanical properties of the cell wall for local cellular growth processes. They are consistent with a conceptual model that explains pollen tube oscillatory growth based on the relationship between turgor pressure and tensile resistance in the apical cell wall. To further confirm the significance of cell mechanics we artificially manipulated the mechanical cell wall properties as well as the turgor pressure. We observed that these changes affected the oscillation profile and were able to induce oscillatory behavior in steadily growing tubes.

Keywords

cell growth; cell mechanics; micro-indentation; plant cell wall; pollen tube; polar growth; tip growth; turgor

Introduction

Plant development is the result of three fundamental processes: cell expansive growth, cell division, and cellular differentiation. Cellular growth in plants involves dramatic changes to the size and the shape of the cell. In most types of animal cells, the mechanical work required to achieve such changes is performed by the cytoskeleton. In plant cells on the other hand, because of the presence of a more or less stiff extracellular matrix, these changes require the concerted action of two mechanical processes: the deformation (stretching) of the existing cell wall and the secretion and deposition of new cell wall material. The cytoskeleton is crucial for the precise targeting of the latter, but the former is thought to be driven by the turgor pressure (Cosgrove, 1993; Geitmann and Ortega, 2009; Lockhart, 1965; Ortega, 2004; Schopfer, 2006).

To mechanically understand plant cell growth, it is helpful albeit simplistic to model the cell as a thin walled pressure vessel. The liquid or gel-like contents is under pressure thus leading to tensile stress in the shell. Hydrostatic pressure is a non-vectorial force, however. Therefore, if the mechanical properties of the shell were uniform and isotropic, the result of a pressure driven expansion would inevitably be a spherical body. However, differentiated plant cells come in all shapes and sizes ranging from simple cylindrical cells (e.g. palisade mesophyll) to star-shaped complex structures (e.g. astro-sclereids). The generation of geometries other than spheres necessitates local differences in mechanical properties of the shell to allow for its localized or uneven deformation. It is, therefore, the pattern of shell extensibility that determines the geometry of the growing body by controlling the spatial distribution of strain on its surface (Green, 1969). Two important mechanical parameters of the shell determine the local generation of strain by an applied tensile stress: 

- Anisotropic extensibility causes the overall symmetry of the body to change by allowing preferential stretching along a particular axis. The plant cell wall is essentially a fiber reinforced composite material with the fibrous component consisting of cellulose microfibrils. Preferential orientation of the microfibrils conveys overall anisotropy to the cell wall (Baskin, 2005).
- Non-uniform distribution of shell extensibility prioritizes expansion of certain regions of the body versus others leading to spatially confined growth (Geitmann and Ortega, 2009). The result is typically a cellular protuberance. Localized secretion of "softer" cell wall material or of agents affecting the properties of the existing cell wall are considered necessary for the generation of such local differences (Smith and Oppenheimer, 2005).

The biomechanics of global cell wall extensibility has been investigated in much detail using tensile testing of plant tissues and organs, but the investigation of local growth events that are spatially confined to subcellular regions requires a single cell approach (Geitmann, 2006a). Regarding such spatially confined cellular growth events, significant advances have been made on the biochemical and biological front. Our knowledge of the agents controlling cell wall extensibility and the molecular mechanisms of cell wall yielding has increased exponentially in the past decade (Cosgrove, 2005). Intriguingly, experimental manipulation of cell wall mechanical properties is able to induce morphogenesis (Pien et al., 2001), but we lack quantitative experimental data that demonstrate the changes to cellular mechanics associated with this process. Cytomechanical approaches will help us understand the physical principles governing plant cell growth and thus might contribute to solving conceptual controversies (Schopfer et al., 2008).

The quantification of physico-mechanical properties at subcellular level is a challenge for micromanipulation and until recently had been thought to be impossible on fast growing plant cells (Messerli and Robinson, 2003). However, the development of micro-indentation and atomic force microscopy have made the mechanical characterization of walls surrounding living cells possible (Geitmann, 2006a);
Geitmann, 2006b; Geitmann and Steer, 2006; Parre and Geitmann, 2005b; Zhao et al., 2005). To study the generation of spatially confined growth events, we chose pollen tubes which are long, cylindrical protrusions formed by germinating pollen grains (Chebli et al., 2007; Zerzour et al., 2005). Their biological purpose is the transfer of the male gametes from the male gametophyte, the pollen grain, to the female gametophyte, located in the ovule of a flower. Pollen tubes exhibit rapid tip growth, a growth pattern that is also characteristic for root hairs and fungal hyphae (Geitmann et al., 2001; Geitmann and Emons, 2000; Heath, 1990). The growth process is unidirectional and confined to the apex of the cellular protrusion (Geitmann and Dumas, 2009; Geitmann and Steer, 2006).

In previous studies we showed that the growing region of pollen tubes spatially coincides with a region of lower cellular stiffness which in turn is a result of differences in the biochemical composition of the cell wall (Bolduc et al., 2006; Geitmann et al., 2004; Parre and Geitmann, 2005a; Parre and Geitmann, 2005b). In the present study we investigated the temporal aspect of the relationship between cellular stiffness and growth activity. To this end we studied the mechanics of two phenomena - the re-initiation of a tubular outgrowth from a sphere-shaped swelling, and the oscillatory change of the pollen tube growth rate.

Material and methods

**Pollen tube growth**

In this study, pollen from different plant species was used since the differences in size and growth dynamics allowed for optimal use in each particular experimental setup. *Papaver rhoes* pollen was used for experiments involving apical swelling and recovery upon cytochalsin D treatment. *Nicotiana tabacum* and *Petunia hybrida* pollen tubes show oscillatory growth with periods in the range of one to several minutes. These species were used for experiments addressing the role of turgor and cell wall properties on the growth rate (sucrose, auxin, pectin methyl esterase data sets). There was no difference in behavior between these two species. *Lilium longiflorum* pollen tubes have a very large diameter and are thus easier to handle in micro-manipulation experiments. This species was used for the quantification of cellular stiffness during growth rate oscillations. The typical oscillation period of this species is between 20 and 50 sec; *Lilium* was also used for investigating role of turgor on oscillation frequency (mannitol data set).

*Papaver rhoes* pollen was obtained from plants grown in the greenhouses of the Montreal Botanical Garden. *Nicotiana tabacum* and *Petunia hybrida* pollen were obtained from plants grown in the Botanical Garden of Siena, Italy. *Lilium longiflorum* flowers were obtained from a local Montreal florist. After collection, pollen was dehydrated in gelatin capsules on anhydrous calcium sulfate overnight and stored at -20°C. Pollen was rehydrated in humid atmosphere for 30 minutes before cultivation. The growth medium for *Lilium* contained 0.16 mM H$_2$BO$_3$, 0.13 mM Ca(NO$_3$)$_2$, 1 mM KNO$_3$, 5 mM MES, 100 mg mL$^{-1}$ sucrose, pH 5.5. Unless specified otherwise, the medium for all other species was composed of 100µg mL$^{-1}$ H$_2$BO$_3$, 300 µg mL$^{-1}$ Ca(NO$_3$)$_2$, 100 µg mL$^{-1}$ KNO$_3$, 200 µg mL$^{-1}$ MgSO$_4$,$\cdot$7H$_2$O, 50 mg mL$^{-1}$ (Papaver) or 120 mg mL$^{-1}$ (Nicotiana, Petunia) sucrose (modified after Brewbaker and Kwack, 1963)). For the mannitol data set, the medium contained 70 mg mL$^{-1}$ sucrose and was complemented with 16 or 32 mg mL$^{-1}$ mannitol to reach the same osmolarity as that of media containing a total of 100 or 130 mg mL$^{-1}$ sucrose, respectively.

For micro-indentation, pollen was brushed on poly-lysine or gelatin coated cover slips, re-hydrated, and covered with drops of liquid growth medium. For cytochalsin D treatment, the liquid growth medium was changed for medium containing 10 µM of the drug once germination had occurred. After 10 min, the slide was submerged into the medium filled sample chamber of the micro-indentor (described below) which effectively diluted the drug to subeffective levels (approximately 0.5 nM).

**Time lapse imaging**

Time lapse imaging for growth rate measurements were carried out on pollen growing on the surface of a thin layer of agarose as described previously (Geitmann et al., 1996). Auxin, pectin methyl esterase, and altered sucrose and mannitol concentrations were administered replacing the liquid layer of medium with medium containing the substance in question. Quantitative analysis of the growth rate was carried out as described previously (Geitmann et al., 1996) or using image acquisition with a Roper fx cooled CCD camera and the tracking function of the ImagePro software (Media Cybernetics, Bethesda, MD, USA).

**Micro-indentation**

Pollen was incubated as described and after germination had occurred, cover slips were submerged in the growth medium containing experimental chamber of the micro-indentor. The design and principles of operation of the micro-indentor have been described previously (Elson et al., 1983; Petersen et al., 1982). The micro-indentation assemblies used here were mounted on either a Zeiss IM 35 inverted light microscope or a Nikon TE2000 inverted microscope. In the experiments reported in this paper, the motor was programmed to execute a single triangular waveform with a velocity of 4 µm s$^{-1}$ and a total amplitude of 10 µm. Time lapse imaging was performed with a Nikon TE2000 inverted microscope equipped with a Roper fx cooled CCD camera. Image acquisition and tracking was done with ImagePro (Media Cybernetics).

**Fourier analysis**

The Fourier analysis technique is based on the fact that a signal can be decomposed into a sum of simple cosine or sine functions with periods that are a multiple of the total recording time. A certain periodicity in the recording is reflected by the large weight (Fourier coefficient) that multiplies the cosine or sine function with the respective periodicity in the Fourier decomposition (Fourier series). The value of these Fourier coefficients as a function of their period (or frequency) is called the Fourier transform. Since the Fourier transform is a complex quantity, we calculate the power of the Fourier series which is a real quantity.

The power $P(q)$ of the fluctuations of the cellular stiffness and the growth rate as a function of their frequency $q$ is computed using

$$P(q) = \frac{\left|F(q)\right|^2}{N}$$

$F(q)$ is the fast fourier transform of the oscillations and $N$ is the number of data points. Prior to the transform, the average of the stiffness or growth rate data sets, i.e. time series, was subtracted from the time series. This standard procedure yields the absence of the D.C. component, i.e. the non-oscillating component of the time series, in the periodogram. Also prior to the transform, some data points were added by interpolation using the cubic spline method. This procedure was necessary to obtain equal time intervals between data points.

To determine the statistical significance of individual peaks, we performed the Fischer Test of significance (Warner, 1998). The fraction of the power of peaks within the biologically relevant range compared to the total power was determined and compared with the critical value for the peaks determined based on the number of data points in the time series.
Results

Deposition of cell wall material is not sufficient to cause cellular growth

In tip growing cells, the material required for the assembly of the elongating cell wall is delivered in highly targeted manner to the growing end of the cell (Bove et al., 2008). This might give the impression that the delivery process drives the actual cellular elongation. To test this hypothesis, we uncoupled the potential other driving force, turgor pressure, from the secretion process. We slightly reduced the osmotic value of the medium surrounding in vitro growing pollen tubes by increasing the amount of sucrose from 10% to 13%. Within approximately one minute, pollen tubes arrested growth while clearly continuing to deliver cell wall material to the apical region of the cell (Fig. 1). This effect was reversible, since upon returning to the original growth medium, cells resumed cellular expansion. The fact that continuing secretion of cell wall material was not able to sustain growth upon increase of the osmotic value of the medium clearly points to the turgor pressure being a prerequisite for cellular expansion.

Initial formation of a cellular protuberance is preceded by local cell wall softening

To assess the spatio-temporal relationship between initiation of a spatially confined growth event and cellular mechanics, we studied the re-initiation of a cylindrical outgrowth from a drug-induced spherical swelling in pollen tubes. Interference with actin functioning by application of 10 µM cytochalasin D resulted in rapid growth arrest and the formation of an approximately sphere-shaped apical swelling. The arrest was reversible, since upon removal of the drug most tubes resumed growth by forming a new, cylindrical outgrowth from the swollen apical region. At the drug concentration used here, this occurred over a period of few minutes to half an hour. Typically, this outgrowth was not formed exactly in the same direction as the original growth direction, but at an angle of up to 90° (Fig. 2). We used this phenomenon to monitor cellular stiffness in different cellular regions.

Figure 1. Turgor induced growth arrest in in vitro growing Nicotiana pollen tube. (A) At t=0 sec the growth medium containing 10% sucrose is replaced by medium containing 13% sucrose. (B) Growth is completely arrested. (C) The apical cell wall shows visible thickening. (D) The cell wall thickening fills the entire apical dome. (E) The medium is replaced with the original growth medium containing 10% sucrose. (F) The apical wall is being stretched. (G) Growth resumes. (H) Parts of the thickened wall are still visible in the now cylindrical, mature part of the tube. Numbers are times in seconds. Arrowheads indicate the plasma membrane in tubes with thickened apical wall. Bar = 5 µm.

Figure 2. Morphology of Papaver pollen tubes treated with cytochalasin D. (A) Normally growing control tube. (B) Swollen apex after 10 minutes of treatment with 10 µM cytochalasin D. (C) Initiation of an outgrowth after removal of cytochalasin D. (D) Typical morphology of an advanced outgrowth from a cytochalasin D induced swelling. Bar = 10 µm.

Figure 3. Temporal changes in cellular stiffness at different locations on Papaver pollen tube during administration and after removal of cytochalasin D. The graph represents the recovery of a typical tube after cytochalasin D treatment. Circles indicate the position of the micro-indenter on the tube. Circle colors correspond to bar colors in the graph. (A) Normally growing pollen tube. The apex is softer than the distal region. (B) The swelling induced by a 10 minute treatment with 10µM cytochalasin D is significantly stiffer than the apex of the normally growing tube or the distal region. (C) Before the swelling becomes visibly asymmetric the stiffness on one side is significantly lowered. (D) The future outgrowth is recognizable. The stiffness in the outgrowth is reduced to the same level as that of a normally growing tube apex. (E) The outgrowth forms into a normally growing tube.
Manipulation of cellular turgor affects oscillatory growth

Pollen tube growth can typically be characterized either as steady or oscillatory (Chebli and Geitmann, 2007). The mode of growth is both species-dependent and influenced by the growth environment and age of the tubes. While a complex network of feedback mechanisms is known to influence oscillatory growth (Messerli and Robinson, 2003; Wilsen and Hepler, 2007), we pointed out previously that all elements of this biochemical oscillator have to act via the mechanics of the cell to effect a change in growth rate (Chebli and Geitmann, 2007; Geitmann, 1999). We postulated that the cellular oscillator centers around an interaction between two forces: the tensile force in the cell wall caused by the turgor pressure and the opposing force resisting cell wall expansion. As long as these two forces are in a steady state during which expansion induced cell wall thinning is compensated by the addition of new cell wall material, a constant strain rate, and hence a constant growth rate results. This steady state can easily be disturbed with fatal consequences. A tensile force significantly higher than the opposing force leads to mechanical failure (rupture), and a significantly increased opposing force can halt growth completely. We postulated that oscillatory rhythms in the growth rate are the result of a milder disturbance to the steady state. We acknowledge that parameters such as oscillation amplitude and frequency are influenced by numerous cellular features and the feedback mechanisms between them. Among these are the cytoskeletal dynamics, ion dynamics, signaling events etc. However, we postulate that to affect growth, all of these regulatory mechanisms ultimately have to act on one of the two forces, the cell wall resistance opposing expansion and/or the turgor generated tensile force.

To demonstrate that experimental data are consistent with this model centered around cell mechanics, we experimentally manipulated the physical properties of the two putative crucial mechanical players of the oscillator: the turgor pressure and the mechanical properties of the apical cell wall. To change the turgor pressure we altered the sucrose or mannitol composition of the growth medium and monitored the effect on oscillation frequency. A sudden increase in sucrose or mannitol concentration caused the pollen tubes to consistently reduce oscillation frequency whereas the inverse was the case when sucrose or mannitol concentrations were suddenly reduced (Figs. 4, 5). For both osmotica, the effect was reversible upon returning to the original growth medium.

Changes in the cell wall properties affect oscillatory growth

To change the mechanical properties of the cell wall in oscillating pollen tubes we administered auxin or pectin methyl esterase (PME). Previous studies had revealed that pollen tube growth is enhanced in the presence of auxin concentrations in the range of 10 µM whereas higher concentrations reduce growth (Chen and Zhao, 2008; Geitmann, 1997). Growth stimulating effects of auxin have been proposed to be mediated in part by the excretion of H⁺ ions into the cell wall through plasma membrane located proton pumps (Hager,
frequency. (A) Addition of auxin significantly increased oscillation frequency. (B) Application of pectin methyl esterase decreased oscillation frequency. (C) In steadily growing pollen tubes auxin induced oscillatory growth. (D) Upon administration of PME, steadily growing pollen tubes exhibited near-regular or regular oscillatory behavior. A,B Petunia, C,D Nicotiana. All cellular responses are typical for both species.

External application of PME on oscillating pollen tubes caused the relatively soft, apical pollen tube wall (Parre and Geitmann, 2005b). This acidification of the apoplast is thought to loosen the wall by breaking acid-labile, load-bearing bonds between cell wall polymers and thus allows the cell to expand more rapidly. We exploited this loosening effect of auxin on cell wall mechanics and applied the hormone at the growth enhancing concentration of 10 µM to oscillating pollen tubes. We observed that oscillation frequency and slow growth rate increased significantly with a reduction in the oscillation period up to two-fold (n=4; Fig. 5, 6A). In some cases pollen tubes burst within minutes after the addition of auxin (n=3). This indicates that cell wall loosening beyond a critical threshold leads to material failure and thus rupture in these cells. PME cleaves the methyl groups from pectins leaving the polysaccharide negatively charged. In the presence of calcium ions, this leads to a gelation of the pectins thus rigidifying primarily the relatively soft, apical pollen tube wall (Parre and Geitmann, 2005b). External application of PME on oscillating pollen tubes caused the oscillation frequency to decrease (Fig. 5, 6B), and in some cases (n=2) growth was arrested completely at 0.1 mg mL$^{-1}$. Higher concentrations of the enzyme are known to consistently arrest pollen tube growth (Bosch et al., 2005; Parre and Geitmann, 2005b). The fact that manipulation of cell wall mechanical properties and turgor pressure influences the oscillation frequency of pollen tube growth is consistent with a crucial role of these parameters in the feedback mechanism governing cellular expansion.

Longitudinal micro-indentation to measure the mechanical properties of lily pollen tubes (Bosch et al., 2005; Parre and Geitmann, 2005b). Values of cellular stiffness as measured by micro-indentation are influenced by the mechanical properties of the cell wall, by the turgor pressure, and by the geometry of the contact area between micro-indenter and pollen tube (Bolduc et al., 2006). We therefore carried out several control experiments that allowed us to ascertain the significance of our experimental data.

Figure 7. Micro-indentation of Lilium pollen tube. (A) The stiffness profile of a growing pollen tube as measured by micro-indentation reveals that the tip of the tube is significantly softer than the shank region. (B,C) Brightfield micrograph of growing pollen tube with micro-indenter positioned at the apex (B) or on the shank region (C). The white circle marks the contact region between micro-indenter stylus and pollen tube, the diameter of the indenter in this experiment was 4 µm. The dark blur is caused by the out-of-focus parts of the micro-indenter. Bar = 10 µm.

Apical cellular stiffness fluctuates during oscillatory growth

If our model is correct and cellular mechanics is the underpinning of growth rate oscillations in pollen tubes, at least one of the two parameters - cell wall or turgor - should undergo oscillatory changes with the same frequency as that of the growth rate. To assess whether temporal changes in cellular mechanics occur during oscillatory growth, we used micro-indentation to monitor the cellular stiffness of growing lily tubes. The spatial distribution of cellular stiffness in pollen tubes of this species (Fig. 7) resembles that of poppy published previously (Geitmann et al., 2004). Values of cellular stiffness as measured by micro-indentation are influenced by the mechanical properties of the cell wall, by the turgor pressure, and by the geometry of the contact area between micro-indenter and pollen tube (Bolduc et al., 2006). We therefore carried out several control experiments that allowed us to ascertain the significance of our experimental data.

Control - Fluctuations in overall turgor pressure

To assess the degree to which overall turgor pressure changes in oscillating pollen tubes we monitored cellular stiffness at a distance of 50 to 100 µm from the tip. At this position, the cell wall mechanical properties were not expected to fluctuate over short time intervals even though slow, gradual changes might occur in relation with the continuous maturing of the wall through callose deposition. Fig. 8

Figure 6. Effect of agents interfering with mechanical cell wall properties on pollen tube growth. (A) Addition of auxin significantly increased oscillation frequency. (B) Application of pectin methyl esterase decreased oscillation frequency. (C) In steadily growing pollen tubes auxin induced oscillatory growth. (D) Upon administration of PME, steadily growing pollen tubes exhibited near-regular or regular oscillatory behavior. A,B Petunia, C,D Nicotiana. All cellular responses are typical for both species.

Alteration of the mechanical cell wall properties can induce oscillatory growth

In a harmonic oscillator in equilibrium, a single mechanical disturbance is sufficient to trigger oscillatory behavior. Therefore, we wanted to find out whether a single, mechanical event would be able to induce oscillatory growth in steadily growing pollen tubes. To this end, we administered auxin to steadily growing pollen tubes. The cellular response ranged from mild to dramatic and could be categorized as follows (with combined effects being possible): (i) a transient increase of the steady growth rate to varying degrees ranging from 2% to 60% (n=10); (ii) a change in growth rate from steady to irregular with occasional pulse-like expansions (n=11); (iii) an induction of regular oscillatory growth (n=3; Fig. 6C).

Similarly, we tested the effect of PME-induced cell wall cross-linking (and thus rigidification) (Parre and Geitmann, 2005b) on steadily growing pollen tubes. As a result the pollen tube growth rate assumed a near-regular or regular oscillatory profile (n=5; Fig. 6D).

2003; Li et al., 2005). This acidification of the apoplast is thought to loosen the wall by breaking acid-labile, load-bearing bonds between cell wall polymers and thus allows the cell to expand more rapidly. We exploited this loosening effect of auxin on cell wall mechanics and applied the hormone at the growth enhancing concentration of 10 µM to oscillating pollen tubes. We observed that oscillation frequency and slow growth rate increased significantly with a reduction in the oscillation period up to two-fold (n=4; Fig. 5, 6A). In some cases pollen tubes burst within minutes after the addition of auxin (n=3). This indicates that cell wall loosening beyond a critical threshold leads to material failure and thus rupture in these cells. PME cleaves the methyl groups from pectins leaving the polysaccharide negatively charged. In the presence of calcium ions, this leads to a gelation of the pectins thus rigidifying primarily the relatively soft, apical pollen tube wall (Parre and Geitmann, 2005b). External application of PME on oscillating pollen tubes caused the oscillation frequency to decrease (Fig. 5, 6B), and in some cases (n=2) growth was arrested completely at 0.1 mg mL$^{-1}$. Higher concentrations of the enzyme are known to consistently arrest pollen tube growth (Bosch et al., 2005; Parre and Geitmann, 2005b). The fact that manipulation of cell wall mechanical properties and turgor pressure influences the oscillation frequency of pollen tube growth is consistent with a crucial role of these parameters in the feedback mechanism governing cellular expansion.

Altering the mechanical cell wall properties can induce oscillatory growth

In a harmonic oscillator in equilibrium, a single mechanical disturbance is sufficient to trigger oscillatory behavior. Therefore, we wanted to find out whether a single, mechanical event would be able to induce oscillatory growth in steadily growing pollen tubes. To this end, we administered auxin to steadily growing pollen tubes. The cellular response ranged from mild to dramatic and could be categorized as follows (with combined effects being possible): (i) a transient increase of the steady growth rate to varying degrees ranging from 2% to 60% (n=10); (ii) a change in growth rate from steady to irregular with occasional pulse-like expansions (n=11); (iii) an induction of regular oscillatory growth (n=3; Fig. 6C).
shows that in oscillating pollen tubes (Fig. 8A) individual stiffness measurements (Fig. 8B) varied only very slightly with the peak-to-peak amplitude being approximately 3% of the mean value. This pattern represents the biological and instrumental noise level as it was indistinguishable from that of the distal region of steadily growing tubes (not shown). Even in the distal region of the tube, the cell wall is sufficiently thin to allow for hydrostatic pressure to influence cellular stiffness measurements. This is easily demonstrated by measuring the cellular stiffness of pollen tubes after the release of turgor through bursting (not shown). Consequently, the absence of measurable oscillatory stiffness variations in the distal region indicates that oscillatory growth does not seem to be accompanied by turgor changes that can be resolved with available methods. This is consistent with observations made with the turgor pressure probe (Benkert et al., 1997).

In this set of experiments the tip of the micro-indenter was kept at the same position between measurements. This eliminated the variability due to slight changes in the geometry of the contact surface between indenter stylus and cell. However, these subtle geometry changes could be caused by the repositioning that will be necessary to measure cellular stiffness at the growing apex.

**Control - Geometry of the contact area**

To assess how small variations in the positioning of the micro-indenter might influence the variability in the measured data, the micro-indenter was moved away from a position on the distal region of the tube by at least 10 µm and subsequently placed back as
precisely as possible to the same position on the median axis before taking the next measurement. Fig. 8C demonstrates that this manipulation increased the noise of the measured values changing the peak-to-peak amplitude from 3% to approximately 5 to 8% of the mean value.

**Fluctuation in apical cellular stiffness**

Since growth is confined to the tip of the pollen tube, putative changes in cell wall mechanical properties during oscillation have to be measured in this cellular region. To monitor cellular stiffness in the growing apex of the tube, the micro-indentor stylus was placed as close as possible to the growing tip. Measurements were taken in approximately 3 sec intervals. Before each measurement the stylus was repositioned to assure a constant distance between stylus and tube apex while the tube was elongating. This repositioning consisted in very small movements (few µm at most). Therefore, noise due to repositioning should be equal to or smaller than the value determined for large repositioning in the previous section. The fluctuations of the apical stiffness in oscillating pollen tubes were considerably larger than the noise in the distal regions of these cells with peak-to-peak amplitudes ranging between 25 to 40% of the mean value (Fig. 8D).

Since data sets for cellular stiffness and growth rate were not acquired simultaneously in this experimental setup, we used Fourier analysis to determine whether the frequencies of the two data sets corresponded for individual cells. In all cases (n=7) the two corresponding periodograms showed at least one common pair of peaks at frequencies within the typical range of oscillation periods (between 15 and 80 sec) (Figs. 8E,F). In all but one case these peaks were determined to be significantly different from white noise. The coincidence of Fourier signals within the biological relevant range strongly indicates that variations in the cellular stiffness are associated with changes in growth rate.

We then attempted to temporally correlate growth rate and apical cellular stiffness. For this purpose both data sets - growth rate and cellular stiffness - had to be obtained simultaneously. Comparison between these simultaneously obtained data sets revealed that troughs in cellular stiffness (= peaks in cellular softness) were consistently followed by the onset of rapid growth phases with typical delays being within a range of 1 to 10 seconds (n=6; duration of each observation >10 minutes). During the rapid growth phases cellular stiffness increased (Fig 8G). Moreover, the height of the peak in cellular softness approximately predicted the height of the subsequent growth peak. Together, the phase lag and the coinciding magnitudes of the corresponding data sets strongly suggest a causal relationship between cell wall mechanics and growth.

**Discussion**

**Mechanics of the morphogenesis of a cellular protuberance**

Global cellular expansion in plant cells is made possible by a controlled yielding of the cell wall under the effect of turgor pressure. Our data reveal the mechanical role of turgor as a deforming force in the spatially confined growth events such as those characterizing pollen tube growth. This is demonstrated by the fact that turgor reduction through increase of the osmotic value of the growth medium caused growth arrest confirming earlier studies (Li et al., 1996). Here we show that secretion of cell wall material during growth arrest is ongoing. This indicates that the delivery of building material is not sufficient to drive cellular expansion in pollen tubes.

To obtain a plant cell shape other than a ballooning sphere, the process of cell wall yielding needs to be anisotropic and/or spatially confined since turgor pressure is a non-vectorial force (Mathur, 2006). Cellular shape changes in walled cells should, therefore, require the non-uniform distribution of cell wall mechanical properties, a principle that had been proposed already more than hundred years ago (Green, 1969; Harold, 2002; Reinhardt, 1892). This principle is a key element of various theoretical models explaining cellular growth in tip growing cells such as pollen tubes, fungal hyphae and root hairs (Dumais et al., 2004; Dumais et al., 2006; Goriely and Tabor, 2003a; Goriely and Tabor, 2003b). The localization of expansins (Baluška et al., 2000), xylolucan endotransglycosylase activity (Vissenberg et al., 2001), and lowered pH (Bibikova et al., 1998) at the sites of bulge formation in root hair forming trichoblasts suggests that the changes to the cell wall biochemistry occur indeed prior to the onset of a spatially confined growth event in this cell type. However, no quantitative mechanical evidence for the spatio-temporal relationship between cell wall mechanical properties and yielding has been brought forward so far. If the physical laws governing the mechanics of pressure vessels apply to growing cells, the formation of a cylindrical protuberance from a spherical structure should require local cell wall softening at the location of the future outgrowth. Our data are the first quantitative mechanical studies that confirm this hypothesis for the generation of a spatially confined growth event. They show clearly that a local reduction of cellular stiffness precedes the onset of a new outgrowth and that only upon reaching a certain threshold in wall extensibility, the formation of a protuberance is initiated. We were able to clearly distinguish the mechanical properties in different cellular regions only few micrometers apart from each other, a distance that is physically impossible to allow for intracellular differences in hydrostatic pressure. Therefore, the parameter responsible for this change in cellular stiffness must be the extensibility of the cell wall.

These findings raise questions about the underlying biochemical events causing the softening. Given that the spatial gradient in cellular stiffness that characterizes growing pollen tubes is to a great degree due to the changing pectin chemistry between the apical region and the shank (Bosch et al., 2005; Geitmann and Parre, 2004; Parre and Geitmann, 2005b), one might expect a similar mechanism to be responsible for the initiation of growth shown here. This would require the local secretion of highly methyl-esterified pectins preceding the growth event. On the other hand, a number of enzymes such as polygalacturonases and pectate lyase have been suggested to manipulate the mechanical properties of the pollen tube cell wall (Bosch and Hepler, 2005). Their non-uniform spatial distribution or local activation could give rise to the observed cell wall softening.

**Mechanical elements of the pollen tube oscillator**

Encouraged by the unequivocal mechanical data for pollen tube initiation we wanted to explore whether rapid growth phases occurring during oscillatory growth are preceded by a softening of the cell wall. Fourier transformation revealed that the temporal changes in apical stiffness show an oscillation component that is related to the changes in growth rate. Comparing simultaneously obtained data sets showed that the apex is softest preceding the rapid phase of the growth cycle and it rigidifies towards the end of a pulse. This suggests that during rapid expansion the cell wall undergoes strain hardening. In this context it is interesting to note that bursting events induced by auxin (this study) or other substances (unpublished) always occurred upon completion of a rapid growth phase. This is consistent with the cell wall being stretched to a point of failure at this time of the growth cycle, after undergoing a rapid expansion. Together, these findings confirm that the cell wall undergoes temporal changes in mechanical properties during the different phases of a growth cycle.

The temporal dynamics of these changes suggest that growth in the tip growing pollen tube follows the same biomechanical laws that apply to plant cell growth in general. Our data are consistent with the notion that cellular growth must be preceded by the relaxation of the cell wall, a concept that is widely accepted for plant cell growth in general (Schopfer, 2006) and that had been suggested to apply to pollen tubes (Bosch et al., 2005). Our findings are not consistent with the alternative hypothesis which proposes rapid growth phases in pollen tubes to be caused by increases in turgor (Zonia and Munnik, 2007; Zonia et al., 2006). The latter would imply that cellular stiffness is...
high preceding a rapid growth phase (due to the increased turgor and resulting tensile stress in the wall) and lower towards the end of a rapid growth episode (due to the reduction in turgor upon "loss-of-stability" in the cell wall). While emphasizing cell wall mechanics, our model does not exclude that changes in turgor can promote growth in other experimental situations, as is evident from recovery of growth after after removal of a high osmotic medium (Fig. 1).

Many aspects of oscillating pollen tube growth have been studied by several labs in the past decade. These studies revealed that numerous cellular processes change with the same frequency as the growth rate albeit not necessarily in phase (Chebli and Geitmann, 2007; Holdaway-Clarke and Hepler, 2003; Messerli et al., 2000; Wilsen and Hepler, 2007). It has therefore become obvious that pollen tube growth is an oscillator that is controlled by the feedback mechanisms acting between the individual elements of the system. Several conceptual models have been helpful in understanding this phenomenon, but most have focused exclusively on the network of biochemical and signaling reactions comprising important cellular players such as ion fluxes, energy production, cytoskeleton, membrane trafficking, and osmotic conditions (reviewed in Chebli and Geitmann, 2007). While these elements are without doubt involved in governing growth, all of them need to manipulate the biophysical properties of the cell to actually affect cellular expansion. It seems therefore reasonable to include the cell wall mechanical properties and the turgor pressure in comprehensive conceptual or mathematical models (Kroeger et al., 2008). We even propose that the steady state relationship between turgor-generated tensile stress and cell wall opposing forces represents a central feature in the feedback network. The observation that a simple mechanical disturbance through the administration of cell wall affecting agents is sufficient to initiate oscillations demonstrates that no rhythm-imposing, biochemical pacemaker is needed to sustain oscillations. Furthermore, the experimental manipulation of both cell wall mechanical properties and turgor affects oscillation frequency, hence confirming the crucial role of the mechanical cellular elements in the dynamics of pollen tube growth.

Significance for mechanical principles governing plant cell growth

Our data show that the initiation of a localized growth activity in plant cells - be it the new cylindrical outgrowth from a sphere shaped swelling or the rapid elongation phase during oscillatory tip growth - is preceded by a local softening of the cell wall. This re-emphasizes the important role the mechanical properties of the plant cell wall plays in the control of cellular growth processes in general. Micro-indentation allowed us for the first time to provide quantitative dynamic information at subcellular level in a living and actively growing plant cell. It confirmed that local differences in biophysical properties are a prerequisite for spatially confined growth leading to complex cellular geometries.

Acknowledgements

Work in the Geitmann lab is supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT), the Human Frontier Science Program (HFSP), and the Canadian Foundation for Innovation (CFI).

References


