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The turgor pressure of growing lily pollen tubes

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Summary. The turgor pressure of growing pollen tubes of the lily (*Lilium longiflorum* Thunb.) has been recorded using a turgor pressure probe. Insertion of the probe's micropipette was routinely accomplished, providing recording periods of 20 to 30 min. Probe insertion did not affect tube growth. The stable turgor values ranged between 0.1 and 0.4 MPa, the mean value being 0.209 ± 0.064 MPa (n = 106). A brief increase in turgor, generated by injection of oil through the pressure probe, caused the tube to burst at its tip. Burst pressures ranged between 0.19 and 0.58 MPa, that is, individual lily pollen tubes do not withstand turgor pressure approaching twice their regular turgor pressure. In contrast, parallel experiments using the incipient plasmolysis technique yielded a mean putative turgor pressure of 0.79 MPa either using sucrose (n = 24) or mannitol (n = 25). Surprisingly, turgor pressure was not significantly correlated with tube growth rate which ranged from zero to 13 μm/min. Nor did it correlate with tube length over the tested range of 100 to 1600 μm. In addition the influence of the medium's osmolality was surprisingly low: raising the external osmotic pressure from 0.36 to 1.08 MPa, with sucrose or mannitol, only caused mean turgor pressure to decline from 0.27 to 0.18 MPa. We conclude that growing lily pollen regulates its turgor pressure remarkably well despite substantial variation in tube growth rate, tube length, and osmotic milieu.

Keywords: *Lilium longiflorum*; Pollen tube; Pressure probe; Tip growth; Turgor pressure.

Introduction

Pollen tubes are well-established model systems for investigating polar cell growth, so-called tip growth, which can be observed throughout the plant and animal kingdoms, e.g., in fungal hyphae, protonemata of mosses and ferns, pollen tubes, and nerve cells (Schnepf 1993, Heath 1995, Derksen 1996, Heidemann 1996, Obermeyer and Bentrup 1996). The common feature of these different cell types is that cell elongation occurs only at the cell tip. In pollen tubes, for instance, secretory vesicles transporting cell wall material fuse exclusively with the plasma membrane at the extreme tip. Tip growth requires spatially and temporally well organized regulation of several cellular components and processes including the cytoskeleton (Pierson and Cresti 1992, Cai et al. 1996), intracellular ion concentrations (Nobiling and Reiss 1987, Obermeyer and Weisenseel 1991, Miller et al. 1992), activity of ion transporters (Obermeyer et al. 1992, Feijó et al. 1995, Malhó et al. 1995), transport of cell wall material (Geitmann et al. 1996), fusion of secretory vesicles (Battey and Blackbourn 1993, Rutten and Knuiman 1993), and finally, turgor pressure must be maintained to allow cell enlargement.

Despite the fact that turgor pressure is generally assumed to act as the driving force for plant cell elongation (Eamus and Jennings 1986, Woods and Duniway 1986, Cosgrove 1993, Amir et al. 1995), its role has not been investigated in pollen tubes. Only a few turgor pressure estimates are available for pollen tubes (Obermeyer and Bentrup 1996). On the other hand, the turgor pressure of tip-growing fungal hyphae has been measured thoroughly using different techniques including incipient plasmolysis (Money 1990, Kaminskyj et al. 1992), psychrometry (Adebayo et al. 1971, Luard and Griffin 1981, Eamus and Jennings 1986, Amir et al. 1992), and the turgor pressure probe (Money 1990, Amir et al. 1995). The turgor pressure probe of Zimmermann et al. (1969) was

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developed to record turgor pressure in individual, living and growing plant cells using an oil-filled micropipette. In fungal hyphae turgor pressure recordings revealed a non-linear relationship between cell growth and turgor, indicating that turgor pressure might not be an important factor for their elongation. Furthermore, hyphae of *Achyla bisexualis* and *Saprolegnia ferax* grew without any detectable turgor pressure (Money and Harold 1993, R. Harold et al. 1996). Therefore, the role of turgor pressure in tip growth of well-investigated fungal hyphae is not as clear as had been previously assumed (Adebayo 1971, Kaminskyj et al. 1992).

In this study we report turgor pressure measurements on pollen tubes of *Lilium longiflorum* growing under various osmolalities. Surprisingly, like fungal hyphae, turgor pressure and pollen tube growth were not linearly correlated.

Material and methods

Plant material

Pollen grains of *Lilium longiflorum* Thunb. were incubated in growth medium (1 mM KCl, 0.1 mM $CaCl_2$, 100 mg of H_3BO_3 per l, pH 5.6) with various amounts of mannitol (2.5% to 10%, w/v) or sucrose (5% to 12.5% w/v) as osmotica. After 1–2 h germinated pollen grains were selected for turgor pressure measurements and mixed with warm 2% agarose (w/v; Sigma, Type VII) in growth medium. The solidified agarose provided mechanical support for the tubes so that the pressure probe micropipette could be inserted without moving the tube. Using this method, media could not be exchanged rapidly enough to measure the instantaneous response of pollen tubes to hyper- and hypoosmotic shock, because the exchange rate of the solutions in agarose was too slow. Pollen tubes were preferentially impaled near the tube base.

Osmotica

The osmolality of the growth media was measured and adjusted with mannitol and sucrose as follows: 150 mosmol/kg: 2.5% (w/v) mannitol, 5% sucrose; 320 mosmol/kg: 5% mannitol, 10% sucrose; 455 mosmol/kg: 12.5% sucrose.

Incipient plasmolysis method

Pollen tubes were pipetted into a perfusable chamber where they settled onto the bottom on a poly-lysine coated cover slip. Growth medium was exchanged by a continuous gradient of increasing osmolality (0.5 to 1.1 osmol of sucrose and mannitol per kg, respectively) while pollen tubes were observed with a video-equipped microscope (Zeiss, Axiovert 135). At the moment when the plasma membrane retracted from the cell wall of the tube tip, the osmolality inside the tube was assumed to be equal to the osmolality of the bathing medium. An aliquot (50 µl) of the solution was withdrawn and assayed for its osmolality (Osmomat 030; Gonotec, Berlin, Federal Republic of Germany). Since the internal osmotic pressure π_i is given by the equivalence of 0.1 MPa and 42 mosmol/kg, the corresponding turgor pressure estimate is given by the difference, $\pi_i - (\pi_e)_0$, where $(\pi_e)_0$ denotes the osmotic pressure of the growth medium. In some experiments, two cytoskeleton inhibitors, depolymerizing actin filaments (10^{-5} M cytochalasin D) and microtubules (10^{-5} M amiprofos-methyl; APM), were added to study the influence of the cytoskeleton on incipient plasmolysis.

Turgor pressure technique

We used a modified version of the pressure probe developed by Zimmermann and co-workers (Zimmermann et al. 1969; see Zimmermann and Steudle 1978, Tomos 1988, Oparka et al. 1991). The turgor pressure of a given cell was transmitted by a glass micropipette filled with silicone oil (AS-4; Wacker Chemie, Munich, Federal Republic of Germany) to a perspex chamber equipped with a pressure transducer (KPY-16; Siemens, Erlangen, Federal Republic of Germany). The probe was mounted on a micromanipulator (Leitz, Wetzlar, Federal Republic of Germany). Micropipettes with an outer tip diameter of about 1–2 µm were pulled from borosilicate glass capillaries (GC 120F-10; Clark Medical Instruments, Reading, U.K.) using a microelectrode puller (Narishige, Tokyo, Japan). When the probe tip was inserted into the pollen tube, cytosol entered the micropipette tip and formed a meniscus against the oil filling of the probe. By appropriate displacement of a metal rod inside the probe the meniscus was held as close as possible to the micropipette tip, so that neither cytosol entered the pipette nor oil was released into the pollen tube. Turgor pressures were measured in pollen tubes grown in medium containing 5, 10, 12.5% (w/v) sucrose or 2.5, 5, 10% (w/v) mannitol as osmotica. Data were recorded on a chart recorder and values were also sampled and digitised every 5 s by a data logger (Micrologger 21X; Campbell Scientific, Shepshed, U.K.). All measurements were performed at room temperature.

Burst pressure measurements

To test the mechanical stability of the pollen tube cell wall after a valid turgor pressure recording, silicone oil was injected into the tube to increase the turgor pressure until the tube burst at the very tip. This pressure was called burst pressure, because it probably represents the maximal pressure that the cell wall could stand.

Statistical analysis

Data are given as means of n individual experiments ± standard deviation (S.D.). To detect significant differences between average turgor pressures an unpaired Student's t-test was used (Sigma Plot; Jandel Scientific, Erkrath, Federal Republic of Germany). The significance level was set to a probability $p = 0.01$. Differences in burst pressure values were analysed in the same way.

Results

Stable turgor pressure was recorded for 10–30 min, when a pollen tube was impaled with the micropipette of the pressure probe. Figure 1 shows that a turgor pressure of about 0.22 MPa was measured for more than 20 min. In all measurements ($n = 106$) turgor pressure did not change significantly with time; no oscillatory turgor changes have been observed. Impaled pollen tubes continued to grow, had a normal clear cap, and cytoplasmic streaming was unaffected.

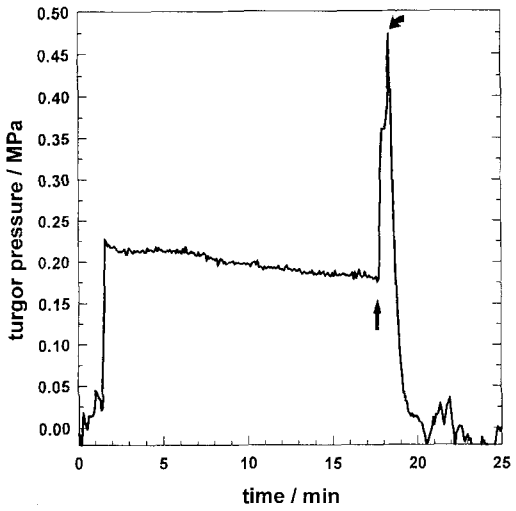

Fig. 1. Recording of turgor pressure of a pollen tube of *Lilium longiflorum* using a turgor pressure probe. In this representative recording a stationary turgor pressure of about 0.22 MPa was measured. At the indicated time (upward arrow) the turgor pressure was experimentally increased by injection of oil into the tube via the micropipette (see Material and methods), until the tube burst at its tip (downward arrow), so that the pressure declined to atmospheric value

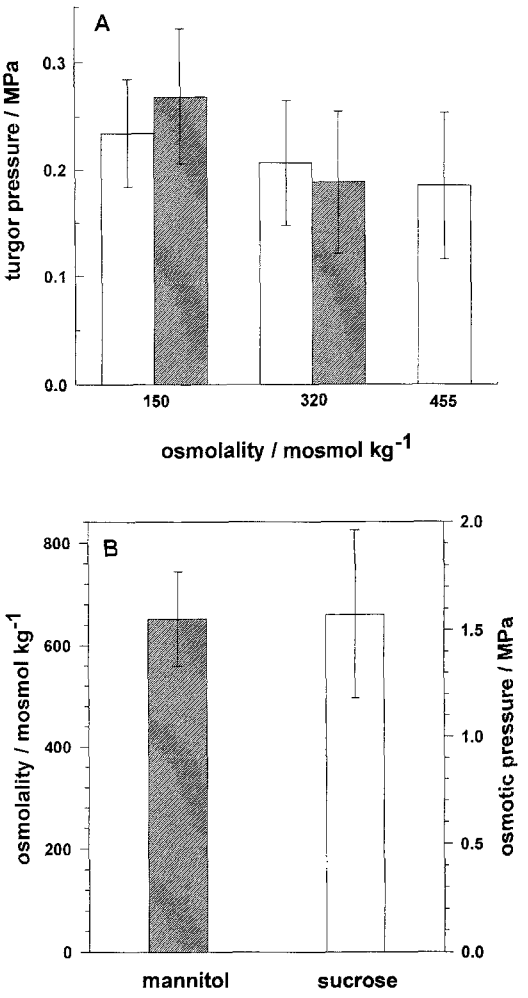

Fig. 2. A Effect of external osmolality upon the stationary turgor pressure of lily pollen tubes measured by the pressure probe. No significant difference in turgor pressures occurred between pollen tubes grown on sucrose (open columns) and mannitol (gray columns), respectively. The turgor pressure did not respond significantly to the sugar concentration (mosmol/kg): 150 vs. 320 sucrose ($p = 0.086$); 150 vs. 455 sucrose ($p = 0.26$); 320 vs. 455 sucrose ($p = 0.025$); 150 vs. 320 mannitol ($p = 0.023$). **B** Incipient plasmolysis observed microscopically on pollen tubes of *Lilium* pollen subjected to increasing concentrations of the given osmolytes. When the protoplast started to retract from the cell wall, aliquots of medium were sampled and their osmolality was measured. Incipient plasmolysis by sucrose ($n = 24$) and mannitol ($n = 25$), respectively, equally requires 650 mosmol/kg (left ordinate). The corresponding osmotic pressure, $\pi_e = 650/42 = 1.55$ MPa, is given by the right ordinate (see text)

No changes in growth rate of the individual pollen tubes was observed when growth was measured before and during turgor pressure recording. The average tube growth rate before impalement was 5.06 ± 2.18 µm/min, during impalement it was 5.52 ± 2.57 µm/min ($n = 10$). Also no difference was detected in pollen tube growth, between growth in agarose solidified medium and liquid growth medium (see Fig. 3 A). Figure 2 A shows the mean turgor pressure of pollen tubes grown and measured in various concentrations of mannitol and sucrose. Recordings were performed in 2 or 3 different concentrations of each osmolyte. The turgor pressure (mean value) only dropped from 0.27 MPa to 0.18 MPa when the external osmotic pressure was increased threefold, i.e., from 0.35 to 1 .08 MPa. The turgor pressure was largely independent of the osmolyte used ($p > 0.01$).

In addition to direct turgor pressure recordings, cell turgor pressure can be estimated by incipient plasmolysis. Therefore, growing pollen tubes were exposed to increasing external osmolality until plasmolysis occurred. Figure 2 B shows putative turgor pressure values for 49 pollen tubes, estimated by the incipient plasmolysis method. Independent of the osmolyte used, pollen tubes which had been growing in 320 mosmol/kg started to plasmolyse at an external osmolality of about 650 mosmol/kg. Plasmolysis actually started at the very tip of the tube. Only at much higher external osmolality was plasmolysis also observed further behind the tip. Since 650 mosmol/kg is equivalent to an osmotic pressure π_e of 650/42 = 1.55 MPa, incipient plasmolysis reflects an internal osmotic pressure $\pi_i = \pi_e = 1.55$ MPa. Putative turgor

pressure is given by the difference, $\Delta\pi = \pi_i - (\pi_e)_0$, where $(\pi_e)_0$, denotes the external osmotic pressure at time zero of the plasmolysis experiment (growth medium); i.e., 0.76 MPa. Thus the plasmolysis-derived turgor pressure is $\Delta\pi = 0.79$ MPa. This putative turgor value obviously exceeds the pressure probe values above by a factor of three or more.

Cytoskeleton inhibitors which depolymerize actin filaments (cytochalasin D) and microtubules (APM) did not decrease the point of incipient plasmolysis (1.83 ± 0.33 MPa versus 1.56 ± 0.39 MPa of the control, sucrose medium). Thus we can conclude that elements connecting the plasma membrane and the cell wall, rather than the cytoskeleton, are responsible for the substantial overestimation of turgor pressure by the incipient plasmolysis method.

Since turgor pressure is assumed to drive cell elongation, growth rates of pollen tubes may at least partly depend on turgor pressure, that is, slowly growing pollen tubes might be expected to have a lower turgor pressure than faster growing tubes. We therefore determined the growth rate and turgor pressure of individual pollen tubes (Fig. 3). The pollen tube growth rate displays high variability; growth rates of individual pollen tubes ranged from 0 to 13 µm/min, independent of the concentration of sucrose or mannitol (Fig. 3 A). Also, no differences in growth rate were detected between solidified and liquid growth medium (Fig. 3 A, see inset). Because of the large deviation, growth rate and turgor pressure were measured on individual pollen tubes. However, no correlation was observed between turgor and growth rate (Fig. 3 B). Even non-growing tubes had a similar turgor pressure to growing tubes. Again, there were also no differences in turgor or growth rate in media of different osmolality.

Assuming a change in concentration of endogeneous osmotica as tube growth continues, longer pollen tubes would be expected to have a lower turgor pressure than shorter ones, but experiments do not support this. Figure 3 C demonstrates that for tubes between 100 to 1600 µm long turgor pressure does not depend on tube length.

We have shown that turgor pressures recorded by the pressure probe were markedly lower than those estimated by incipient plasmolysis. To clarify this discrepancy, we determined the maximal turgor pressure the cell wall can withstand before the tube bursts. For this test, oil was injected into the pollen tube to increase the turgor pressure. At the critical pressure the tubes burst and turgor pressure relaxed to the

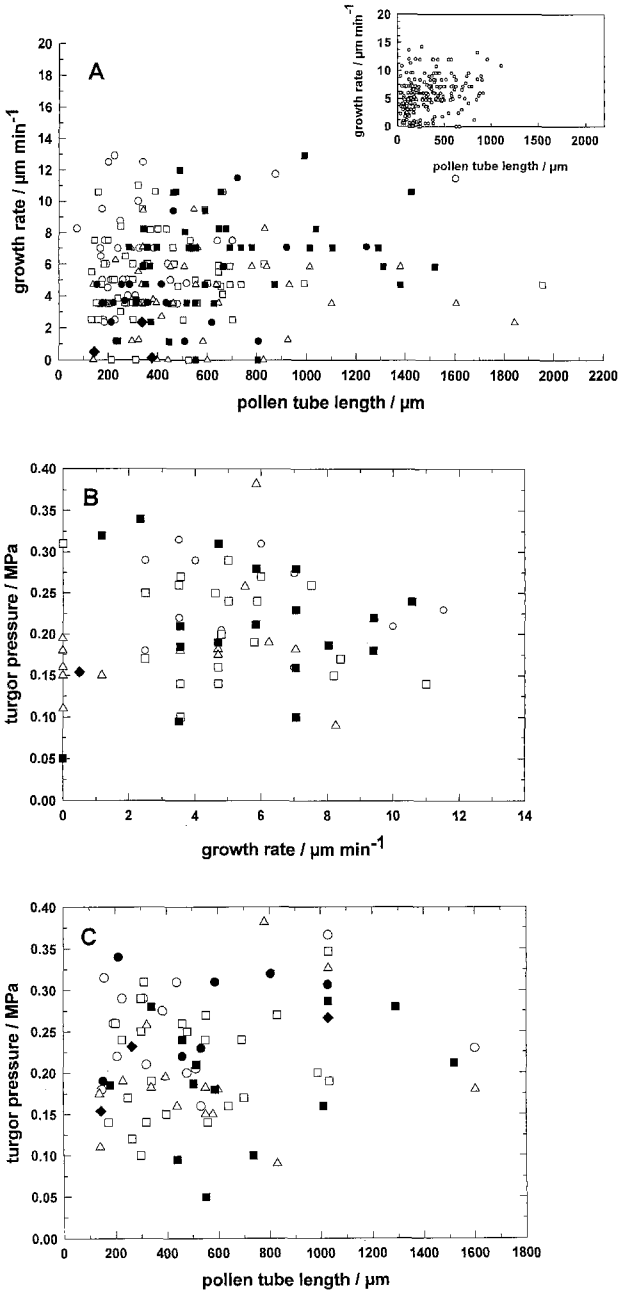

Fig. 3. **A** Growth rate of lily pollen tubes of different tube length (abscissa) and different concentrations of sucrose (open symbols) and mannitol (filled symbols) dissolved in agarose solidified medium. **Inset** Growth rate of lily pollen tubes in liquid sucrose growth medium (320 mosmol/kg). **B** Turgor pressure of lily pollen tubes recorded by the pressure probe plotted as a function of growth rate (abscissa) and medium osmolality (mosmol/kg). Note, that, even without visible growth, tubes exhibited a significant turgor pressure. **C** Turgor pressure of lily polen tubes as a function of tube length. The experiments were performed in different osmolalities. 150 (○, ●), 320 (□, ■), 455 (△), and 600 (◆)

atmospheric value (cf. Fig. 1). Tubes usually burst at their tips where the newly synthesised cell wall is more fragile than that in more distal regions. It should

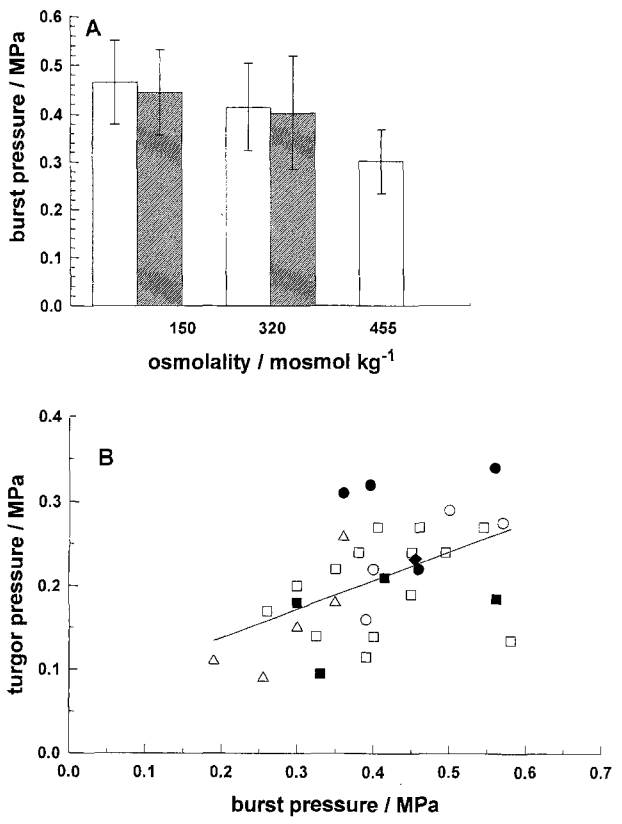

Fig. 4. Limiting turgor pressure (burst pressure) of growing lily pollen tubes subjected to an experimental turgor pressure increase via the pressure probe (see Fig. 1) as a function of **A** osmolality of the external medium and **B** turgor pressure. **A** Burst pressures were measured in sucrose (open column) and mannitol (gray column), respectively. A significant ($p < 0.01$) decrease in burst pressure with increasing osmolality only occurred in pollen tubes exposed to 150 vs. 455 mosmol sucrose per kg ($p = 0.009$). **B** Stationary turgor pressure against the burst pressure of a given pollen tube for different sugar concentrations (mosmol/kg): 150 (○, ●), 320 (□, ■), 455 (△), and 600 (◆). The data were tentatively fitted by a regression line with $f(x) = 0.343x + 0.069$ ($r^2 = 0.256$)

be noted that preferential bursting at the tip indicates that micropipette insertion does not per se cause a leak, otherwise the tube would rupture preferentially at the point of impalement when pressure is increased experimentally.

Figure 4 A shows the mean burst pressure of pollen tubes as a function of external osmolality. Pollen tubes burst at pressures between 0.19 MPa and 0.58 MPa. This was about twice the normal turgor pressure of the respective pollen tubes (Fig. 4 B), indicating that pollen tube cell walls could only hold pressures below 0.6 MPa. Burst pressures were independent of the osmoticum used. Only slight correlation between burst pressure and osmolality was observed. The pressure decreased with increasing osmolality, but significant values ($p < 0.01$) were only found in pollen tubes grown in media of different sucrose concentrations (150 to 455 mosmol/kg, $p = 0.009$). Burst pressures decreased from 0.46 MPa in low osmolar to 0.3 MPa in high osmolar medium. With mannitol only insignificantly smaller changes in burst pressure have been observed.

Discussion

In this study the turgor pressure of pollen tubes of *Lilium longiflorum* was measured directly with a cell turgor pressure probe. Continuous recording was feasible for 20 to 30 min. With careful insertion of the pressure probe micropipette tube morphology was unaffected and, most significantly, tubes continued to grow at their normal, though individual rate (Fig. 3 B).

Average turgor pressure was 0.209 ± 0.064 MPa ($n = 106$), more or less independent of the osmolyte used and the osmolality of the growth medium. Turgor pressure remained in the narrow range from 0.18 MPa to 0.27 MPa, while the osmotic pressure of the growth medium varied from 0.36 MPa to 1.08 MPa. Estimates of pollen-tube turgor pressure by the incipient plasmolysis method yielded a mean value of 0.79 MPa (Fig. 4). This exceeds the pressure-probe values by a factor of three or more (cf. Fig. 3 B, C). Thus, we doubt that this is a reliable measure of cell turgor pressure. Considerable forces may be necessary to overcome linkages between plasma membrane and cell wall, resulting in overestimation of turgor pressure. For instance, a cytoskeleton-bound linkage between the plasma membrane and cell wall might be mediated by vitronectin, as identified by Wang et al. (1994). Indeed pollen tubes exhibit a particularly elaborate cytoskeleton network (Pierson and Cresti 1992, Lancelle and Hepler 1991, Tang et al. 1989). However, in our study the addition of cytoskeleton inhibitors that depolymerize actin filaments (10^{-5} M cytochalasin D) and microtubules (10^{-5} M APM) did not significantly affect the external osmotic pressure required for incipient plasmolysis. (We measured $\pi_e = 1.83 \pm 0.33$ MPa versus 1.56 ± 0.39 MPa of the control sucrose medium.) Thus other mechanisms linking plasma membrane and cell wall in the *Lilium* pollen tube seem to be responsible for the obvious overestimation of turgor pressure by the incipient plasmolysis technique. The most cogent evidence for our suggestion that the plasmolysis-derived

turgor pressure of about 0.79 MPa is an overestimation rests on the finding that *Lilium* pollen tubes invariably burst if the turgor is experimentally increased beyond a limiting pressure of about 0.19 to 0.58 MPa (cf. Figs. 3 and 4). In most cases tubes burst when the pressure was about twice the turgor pressure of the respective tube.

A slight decrease in burst pressure, as observed in pollen tubes growing under increased osmolality (Fig. 4) has also been reported for fungal hyphae of *Saprolegnia ferax* by Money and Harold (1993). The authors argued that the tensile strength of the tip cell-wall could be regulated by a cell wall softening process, probably based on endocellulases. More recently R. Harold et al. (1996) assumed that the rate of cell wall hardening depends on the stress exerted upon the nascent cell wall by hydrostatic pressure. This explanation is offered to explain variable turgor pressure in the hyphae. In pollen tubes, however, turgor pressure remained almost constant when tubes were grown in media of different osmolality. Therefore, we cannot explain the decrease in burst pressure by a simple hydrostatic model.

Turgor pressure and pollen tube growth

Turgor pressure is generally assumed to be the driving force of cell enlargement (Eamus and Jennings 1986, Woods and Duniway 1986, Mohr and Schopfer 1992, Cosgrove 1993, Derksen 1996). Direct dependence of growth rate on turgor pressure might therefore be expected. However, in our study of lily pollen tubes no correlation was found between these two parameters. One may therefore argue that turgor pressure is essential, but does not regulate tip growth.

In some fungi measurable turgor pressure may not even be necessary for cell expansion. Money and Harold (1992, 1993), F. Harold et al. (1995), and R. Harold et al. (1996) applied a turgor pressure probe to hyphae of *Achyla bisexualis*. Surprisingly, they could not detect any turgor pressure, although the hyphae continued growing. Another member of this family, *Saprolegnia ferax,* also produced normal hyphae under conditions, when no turgor pressure could be detected.

Oscillatory growth of lily pollen tubes was observed by Pierson et al. (1996). Alternating periods of slow and fast growth are presumed to be a result of oscillation of Ca^{2+} entry at the tube tip, which results in a periodic pattern of cell wall formation. Such an oscillatory growth model implies an oscillatory increase in cell volume which would, in turn, require or cause periodical changes in turgor pressure. In our study we never observed oscillatory changes in turgor pressure exceeding the resolution limit (noise) of our recordings (approx. 0.005 MPa). Presumably, increased cell volume is immediately compensated by uptake of osmotically active compounds. Also, changes in the rate of tube-tip growth with time do not seem to be evoked by corresponding changes in cell turgor (cf. Fig. 3 B).

Turgor pressure regulation

In *Lilium* pollen tubes turgor is more or less constant despite substantial variation in external osmolality. For experimental reasons, our present turgor recordings were performed in media of constant osmolality, the osmolality being set when the pollen tubes started growing. Therefore, we did not investigate osmotic adaptation of the growing tubes to an osmotically changing milieu. During incubation in growth medium pollen tubes may synthesise osmotically active compounds (e.g., polyamines; Chibi et al. 1994) or degrade storage products to keep the turgor pressure largely independent of the milieu's osmolality, as documented by the present study. Degradation of starch granules and lipid bodies has been observed during germination and growth of pollen tubes, to provide energy and cell wall material (Miki-Hiroshige and Nakamura 1982). These metabolic processes might well be involved in long-term turgor-pressure regulation in *Lilium* pollen. Alternatively, turgor-pressure regulation could be achieved by ion fluxes through ion channels, as is well known from stomatal guard cells (cf. Blatt 1991, Hedrich 1994). So far, no ion channels involved in the regulation of turgor pressure of pollen tubes have been identified (Feijó et al. 1995). However, instantaneous, transient changes in membrane potential have been observed in *Lilium* (Obermeyer unpubl. data), possibly indicating sufficient ion release to regulate turgor pressure, as described for *Chara* cells (Thiel et al. 1993).

Turgor sensing could rely upon stretch-activated Ca^{2+} channels in the pollen-tube plasma membrane. This ion channel species has already been proposed as a mediator of the entry of Ca^{2+} ions at the extreme tip of the pollen tube (Feijó et al. 1995) as well as in fungal hyphae (Heath 1995). Garill et al. (1992) claimed that stretch-activated ion channels permeable to Ca^{2+} ions are activated in the plasma membrane of hyphal tips of *Saprolegnia ferax* following the application of

pressures of 0.004 MPa (30 mm Hg, suction through the patch pipette). In guard cells of *Vicia faba* the open probability of ion channels increased upon suction from about 0.002 to 0.02 MPa (Cosgrove and Hedrich 1991).

Such putative channel activating pressures are below the resolution limit of our turgor pressure probe (0.005 MPa). Furthermore, we never observed pressure transients or changes in pressure noise sufficient to activate stretch-activated channels.

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