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Osmoregulation in *Lilium* pollen grains occurs via modulation of the plasma membrane H^{+} ATPase activity by 14-3-3 proteins[1]

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Abstract

To allow successful germination and growth of a pollen tube, mature and dehydrated pollen grains take up water and have to adjust their turgor pressure according to the water potential of the surrounding stigma surface. The turgor pressure of pollen grains of *Lilium longiflorum* Thunb. was measured with a modified pressure probe for simultaneous recordings of turgor pressure and membrane potential to investigate the relation between water and electrogenic ion transport in osmoregulation. Upon hyper-osmolar shock the turgor pressure decreased and the plasma membrane (PM) hyperpolarizes in parallel whereas depolarization of the PM was observed with hypo-osmolar treatment. An acidification and alkalinisation of the external medium was monitored after hyper- and hypo-osmotic treatments, respectively, and pH changes were blocked by vanadate indicating a putative role of the PM H^+ ATPase. Indeed, an increase in plasma membrane-associated 14-3-3 proteins and an increase in PM H^+ ATPase activity were detected in pollen grains challenged by hyper-osmolar medium. We therefore suggest that in pollen grains the PM H^+ ATPase via modulation of its activity by 14-3-3 proteins is involved in the regulation of turgor pressure.

Introduction

Plant cells, surrounded by a rigid cell wall have an internal hydrostatic pressure, the turgor pressure. The turgor pressure depends on the concentration gradient of osmolytes across the plasma membrane and therefore, changes in osmolytes lead to turgor-driven movements of entire plant organs, e.g. leaves of *Samanea saman* (Moran, 2007), *Dionea* trap leaves (Hodick and Sievers, 1989) or changes in the cell shape of guard cells (Roelfsema and Hedrich, 2005). Especially in guard cells turgor pressure values can reach up to 0.12 MPa (12 bar, Franks et al., 1998). Turgor pressure values can be estimated indirectly by observing plasmolysis or directly measured by impaling a pressure probe into the living plant cell still surrounded by an intact cell wall (Hüsken et al., 1978). The use of a pressure probe not only allows the monitoring of the turgor pressure over a time period but also determination of the hydraulic conductivity of the plasma membrane (L_P), the elastic modulus of the cell wall (ε) and measurements of the permeability of substances across the plasma membrane (σ) (Boyer, 1995; Steudle, 1993; Zimmermann, 1989; Tomos and Leigh, 1999). In guard cells changes in the activity of a number of ion transporters in the plasma membrane lead to a change in the concentration gradient of osmolytes and therefore, water following its potential gradient will either increase or decrease the turgor pressure, thus leading to opening and closing of the stomata, respectively (Roelfsema and Hedrich, 2005; Pandey et al., 2007). On the other hand, environmental changes, e.g. in the water potential of the soil will affect the turgor pressure of root cells which are responding to maintain their turgor pressure (Frensch and Hsiao, 1994). In hyper-osmotically stressed *Arabidopsis* root cells, a rapid, transient hyperpolarization of the plasma membrane was observed while during recovery of turgor pressure an uptake of ions (K^+, Cl^-, Na^+) was measured (Shabala and Lew, 2002). Furthermore in suspension culture cells, hyper- and hypo-osmotic shock caused corresponding changes in turgor pressure and also induced an acidification and alkalinisation of the external medium, respectively, mainly carried by modulation of the plasma membrane H^+ ATPase activity via 14-3-3 protein binding (Reuveni et al., 1987; Curti et al., 1993; Babakov et al., 2000; Felix et al., 2000; Kerkeb et al., 2002). This correlation between changes in turgor pressure and ion transport processes is widespread and can be observed in different plant organs, but also in algae and fungi (Shabala et al., 2000; Lew, 1996; Lew et al., 2004; Bisson and Beilby, 2008). One may therefore assume that ion and water transport in plant cells are connected to each other not only by affecting physical parameters important for both transport processes (e.g. osmolyte/ion concentration and pressure), but due to active regulation and signal cross talk between both processes as has been shown in yeast cells (Muzzey et al., 2009).

Pollen grains (PGs), the male gametophytes of higher plants, are exposed to dramatic changes in environmental water potential. In the last stage of maturation, pollen grains are dehydrated and are then transferred to a stigma surface on which they will immediately take up water to re-hydrate. Before germination occurs, the internal ion concentrations, pH and also the turgor pressure have to be adjusted to allow the germination and growth of a pollen tube through the pistil tissue towards the ovules (Feijó et al., 1995). PGs do not "foresee" the water potential of the stigma on which they are going to land and interestingly, *in-vitro* cultivated PGs of the same species are capable to germinated in media containing various concentrations of osmolytes, e.g. lily pollen grains can germinated in 5 to 12 % sucrose

corresponding to 150 and 450 mosmol kg^{-1}, respectively, with a more or less constant turgor pressure independent from the medium osmolality (Benkert et al., 1997). On the other hand, growing pollen tubes (PTs) are very sensitive to osmotic changes in the medium and stopped growth or increased the volume of the tip region (tip swelling) (Pierson et al., 1994; Zonia and Munnik, 2004). The turgor pressure of lily PTs stays more or less constant (0.21 ± 0.06 MPa) and is independent from tube growth rates, tube length and medium osmolality (Benkert et al., 1997). Artificial increases of turgor pressure by the pressure probe immediately stopped growth and further increase resulted in bursting of the tube tip with a "burst pressure" approximately twice the previous pressure, indicating a fine-regulated balance between turgor pressure and cell wall strength at the tube tip which is necessary for successful growth of pollen tubes (Winship et al., 2010). However, pollen tubes and grains are surrounded by an electrical field which is generated by localized in- and effluxes of ions (Holdaway-Clarke and Hepler, 2003; Michard et al., 2009), thus changing the cytosolic osmolyte concentration. Additionally, metabolic activity during pollen tube growth will also contribute to variations in osmolyte concentrations which will result in changes in the cytosolic water potential and therefore, in water flux amount across the plasma membrane or even water flux direction. Therefore, one may assume an osmoregulation mechanism taking place in pollen grains which (i) somehow senses the osmotic conditions on the stigma surface, (ii) allows an influx of water to account for the volume increase in germination and tube growth, and (iii) adapts the turgor pressure to grow a pollen tube without bursting. Several cellular components that are involved in osmoregulation as demonstrated for other plant cells, were detected in pollen, too: ion channels (Obermeyer and Blatt, 1995; Fan et al., 1999; Mouline et al., 2002; Griessner and Obermeyer, 2003; Frietsch et al., 2007; Sze et al., 2004), a PM H$^+$ ATPase (Obermeyer et al., 1996; Certal et al., 2008; Pertl et al., 2009) and 14-3-3 proteins (Pertl et al., 2005). In this study, invasive and non-invasive techniques were used to investigate whether pollen grains are capable to adjust and regulate their turgor pressure and which transport activities are affected by turgor changes and therefore, are involved in osmoregulation of pollen grains.

Results

Pollen grain turgor pressure and membrane potential

Immediately after impalement with the micropipette of the pressure probe, a stable turgor pressure of 0.317 ± 0.07 MPa (n = 17) was recorded in lily pollen grains bathed in standard medium (298 mM mannitol, Fig. 1). Exposing the pollen grains to various external mannitol concentrations which still allow germination and tube growth (Benkert et al., 1997), resulted in an adaptation of the turgor pressure to the new osmotic condition. The turgor pressure decreased to a lower value when the measuring chamber was perfused with a medium of higher osmolality (398 mM mannitol) or increased upon perfusion with a lower osmolar medium (198 mM mannitol, Fig. 1A). The changes in turgor pressure were always reversible in the range of media used for perfusion (ca. 50 – 520 mosmol kg^{-1}) and lily pollen grains behave like a linear osmometer (Fig. 1B) indicating a reflection coefficient close to 1 for mannitol. At least no changes in the concentration of mannitol during the experiment due to an uptake by the pollen grains were detectable. A similar linear osmotic behavior was observed when lily pollen grain protoplasts were exposed to various mannitol

concentrations (Sommer et al., 2007). The osmotic potential of the cytosol could be estimated as 580 mosmol kg^{-1} from the regression line (Fig. 1B). During some measurements, the turgor pressure was manually changed by the pressure probe and its relaxation was recorded (Fig. S1) to determine the hydraulic conductivity of the plasma membrane. The hydraulic conductivity (L_P) was calculated from the half-time of the turgor pressure relaxations according to Steudle (1989) and Zimmermann (1989) giving $4.89 \pm 5.20 \ 10^{-8}$ m $MPa^{-1}s^{-1}$ (n = 14) which corresponds to an osmotic permeability coefficient (P_{os}) of ca. 7.4 µm s^{-1} which is well in the range of P_{os} values determined by swell assays of lily pollen grain protoplasts (6.6 µm s^{-1}, Sommer et al., 2007).

To monitor effects on electrogenic transport processes across the plasma membrane in parallel to turgor pressure changes, simultaneous measurements of the turgor pressure and the membrane potential were performed by impaling the pollen grain with a micropipette that was partially filled with an electrolyte (1 M KCl) to ensure contact with an Ag/AgCl wire connected to a voltage amplifier (Fig. 2A). A glass capillary inside the micropipette allowed an electrical contact between the cytosol and the electrolyte-filled part of the pressure probe thus measuring the membrane potential of the impaled pollen grain (Zhu, 1996). The pollen grain turgor pressure values measured by the modified pressure probe were similar to those recorded with the original pressure probe. Perfusion with a hypo-osmotic medium (198 mM mannitol) increased the turgor pressure and simultaneously depolarized the plasma membrane (Fig. 2 B) whereas upon perfusion with a hyper-osmotic medium (398 mM mannitol) the turgor pressure decreased as expected and the plasma membrane was hypolarized (Fig. 2C). For each measured pollen grain, the change in medium osmolality (Δosmol) was plotted against the corresponding change in membrane potential (ΔV_M) giving a linear relation with hyper-osmotic media causing hyperpolarization and hypo-osmotic media resulted in depolarization of the plasma membrane (Fig. 2D). These deflections of the membrane potential indicate the presence of an electrogenic transport process that is coupled to or responds to turgor pressure changes in lily pollen grains.

External pH changes upon osmotic stress
Pollen grains incubated in *in-vitro* germination medium extrude H^+ and acidify the external medium (Southworth, 1983; Rodriguez-Rosales et al., 1989). The acidification of the external medium is inhibited by vanadate and promoted by fusicoccin, two well-characterized modulators of the plasma membrane H^+ ATPase in plants. Therefore, monitoring a vanadate-sensitive acidification of the external medium provides a non-invasive method to record the PM H^+ ATPase activity during various osmotic treatments. Under standard osmotic conditions (298 mM mannitol) an acidification of the medium was recorded for one hour after suspending the lily pollen grains in the incubation chamber (Fig. S2). Addition of 500 µM Na^+ vanadate clearly inhibited acidification (Figs. 3B and S2) whereas the addition of 1 µM fusiccocin stimulated the H^+ extrusion (Fig. S2). Immediately after the exposure to hypo- or hyper-osmotic solutions transiently changes in the acidification rate could be observed leading to a slight alkalinisation or to a faster acidification during the first 100 s after osmotic stress (Fig. 3A). Addition of an iso-osmotic solution did not affect the previously recorded acidification rate (Fig. 3A). The observed alkalinisation as well as the acidification after the osmotic stress were both sensitive to vanadate. In the presence of 500 µM Na^+ vanadate almost no changes in

the external pH were detectable after osmotic shock (Fig. 3B). Thus, the activity of the PM H$^+$ ATPase might be responsible for the pH as well as for the membrane potential changes induced by osmotic challenges. One might therefore assume that during exposure to hyper-osmotic conditions the PM H$^+$ ATPase activity is increased resulting in a hyperpolarization of the plasma membrane and a higher acidification rate of the external medium and on the contrary, a hypo-osmotic stress reduces the PM H$^+$ ATPase activity leading to a depolarization of the plasma membrane and a transient alkalinisation of the medium.

Plasma membrane-associated 14-3-3s and PM H$^+$ ATPase activity

To test whether the up- or down regulation of the PM H$^+$ ATPase activity is mediated by 14-3-3 proteins, the abundance of plasma membrane-associated 14-3-3 proteins after different osmotic treatments was detected. Intact pollen grains were suspended in standard germination medium M (298 mM mannitol) for 15 min and were then incubated in iso-, hypo- and hyper-osmolar medium M for another 15 min before plasma membrane vesicles were isolated by sucrose step gradient centrifugation. The amount of PM H$^+$ ATPases was unaffected by the osmotic treatment showing similar band intensities after immunodetection in the crude membrane (MF) as well as in the plasma membrane-enriched fraction (PMF, Figs. 4A and 4B, left panel). The amount of 14-3-3 proteins in the soluble protein, cytosolic fraction (CF) was also not affected upon osmotic treatment whereas membrane-associated 14-3-3 proteins clearly show a decrease, no effect and an increase in the crude membrane fraction (MF) after hypo-, iso- and hyper-osmolar treatments, respectively (Fig. 4A). In isolated plasma membrane vesicles, 14-3-3 proteins were detected with 2 different antibodies, MUP5 and MUP15, and showed changes in signal intensities upon osmotic treatments (Fig. 4B). Compared to controls (iso-osmolar treatment) hypo- and hyper-osmotic shock resulted in less or more plasma membrane-associated 14-3-3 proteins (Fig. 4B) which was confirmed in 3 independent PMF preparations after osmotic shock treatment. The Coomassie-stained protein gel (Fig. 4C) serves as a loading control to demonstrate the equal amounts of protein loaded on each lane. In addition, similar results were observed when 14-3-3 proteins in the PMF were detected with antibodies against *Arabidopsis* 14-3-3s (at-82, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and *Hordeum vulgare* 14-3-3s (Hv14-3-3A, Hv14-3-3B, Hv14-3-3C, Agrisera, Vännäs, Sweden and gifts from Bert de Boer, data not shown).

Additionally, an increase in vanadate-sensitive ATP hydrolysis was detectable after hyper-osmolar treatment indicating an activation of the PM H$^+$ ATPase activity in plasma membrane-enriched vesicles (Tab.1). Compared to the ATPase activity under iso-osmotic conditions the vanadate-sensitive ATPase activity decreased by 30 % after hypo-osmotic shock whereas upon hyper-osmotic treatment the ATPase activity increased by 26 %, probably due to the interaction with 14-3-3 proteins.

Discussion

Generally, pollen tubes growing in *in-vitro* culture are very sensitive to external perturbations and stop growth immediately or even burst at their tips. Small changes in external parameters like pH, K$^+$ and Ca^{2+} concentrations or osmolality lead to temporal cessation of growth or change in growth rate oscillations which are often accompanied by morphological changes of the tube tip region (Pierson et al., 1994; Malhó and

Trewavas, 1996; Messerli and Robinson, 2003; Zonia and Munnik, 2004; Zerzour et al., 2009). This flexibility to react to small environmental changes may help the pollen tube to grow towards the ovary as fast as possible despite varying conditions along the style. In particular, pollen tubes have to adapt their cell wall strength to osmotic changes which would otherwise lead to tube bursting (Zerzour et al., 2009; Winship et al., 2010). The mechanisms and pathways, how pollen tubes sense or regulate their turgor pressure, osmolyte concentrations and water fluxes are still not well characterized. Pollen grains also need to adapt to osmotic conditions of their environment, especially after landing on a receptive stigma when they first take up water and have to adjust their internal ion and osmolyte concentrations to build up a turgor pressure that drives the germination of the tube but still is balanced with the cell wall strength to prevent bursting of the germinating grain (Winship et al., 2010). In contrast to growing pollen tubes which reflect osmotic changes irreversibly in their tube morphology, most manipulations, osmotic changes in particular (Fig. 1), are reversible in pollen grains making them a suitable experimental system for first investigations of the principles of osmoregulation in pollen.

The turgor pressure immediately responded to osmotic changes and lily pollen grains react like a linear osmometer using mannitol as an osmolyte (Fig.1). Simultaneously with the turgor pressure the membrane potential difference (V_M) also changed resulting in a hyper- and depolarization of the plasma membrane after hyper- and hypo-osmotic shock, respectively (Fig 2D). Similar changes in V_M upon osmotic treatments have been reported from other plant cells: in broad bean mesocarp cells V_M was sensitive to tugor pressure changes (Li and Delrot, 1987) and *Arabidopsis* root cells showed a transient hyperpolarization after addition of 100 mM mannitol (Shabala and Lew, 2002). A correlation between turgor pressure and changes in V_M or in electrogenic transport across the plasma membrane was also reported from algal and fungal cells (Bisson and Kirst, 1995; Lew et al., 2004; Lew and Levina, 2007; Bisson and Beilby, 2008). In *Arabidopsis* and carrot cell culture cells (Reuveni et al., 1987; Curti et al., 1993) as well as in leaf mesophyll tissue of beans (Shabala et al., 2000), an increase in (vanadate-sensitive) proton extrusion was detected after turgor pressure decreases by external addition of mannitol indicating an involvement of the plasma membrane H^+ ATPase in the turgor pressure-induced changes in V_M. By measuring the pH changes (acidification or alkalinization) of the germination medium during *in-vitro* culturing of pollen, the activity of the PM H^+ ATPase can be recorded in a non-invasive way (Southworth, 1983; Tupy and Rihova, 1984; Rodriguez-Rosales et al., 1989). In lily pollen cultures a vanadate-sensitive and fusicoccin-stimulated acidification is detectable (Fig. S2) and upon hyper- and hypo-osmotic treatment an acidification and alkalinization, respectively, was observed (Fig.3). Both changes in external pH could be inhibited by vanadate indicating that the changes in V_M are caused by changes in the electrogenic H^+ transport of the PM H^+ ATPase which are also reflected in the increase or decrease of the ATP hydrolysis activity of the PM H^+ ATPase. In general, the activity of PM H^+ ATPase is modulated by the binding of 14-3-3 proteins to the C-terminal autoinhibitory domain which has been reversibly phosphorylated (see Duby and Boutry, 2009 and references therein). 14-3-3 proteins have been detected in almost all cellular compartments of lily pollen grains and tubes (Pertl et al., 2005) and an increase in plasma membrane-associated 14-3-3 proteins is observed after treatment of PGs with hyper-osmotic medium. A similar correlation between hyper-osmolar treatment, activated H^+ transport or H^+ efflux

and abundance of 14-3-3 proteins at the plasma membrane was observed in suspension-cultured cells of tomato (Kerkeb et al., 2002) and sugar beet (Babakov et al., 2000). In contrast to Kerkeb et al. (2002) who observed an increase only in H^+ transport but not in ATP hydrolysis activity upon hyper-osmotic shock with NaCl, the H^+ transport and the ATP hydrolysis of the PM ATPase in lily pollen grains are both stimulated by hyper-osmotic treatment with mannitol.

Conclusion

These data support the hypothesis that short-term osmoregulation in plants cells including pollen grains, involves a modulation of the PM H^+ ATPase activity by interactions with 14-3-3 proteins (Fig.5). Upon hyper-osmolar shock, the turgor pressure decreases and a hyperpolarization of the plasma membrane can be measured due to an activation of the PM H^+ ATPase via 14-3-3 interaction. Consequently, a membrane potential more negative than the reversal potential of K^+ allows a higher K^+ influx which in turn promotes water uptake to re-adjust the turgor pressure in the long term. On the other hand, a hypo-osmotic shock increases the turgor pressure and down-regulates the PM H^+ ATPase activity via dissociation of 14-3-3 proteins. The resulting depolarization of the plasma membrane enables K^+ efflux followed by water efflux and the turgor pressure finally decreases again to the previous value. The short-term effects on turgor pressure and H^+ ATPase activity have been measured in this study whereas the hypothized changes in K^+ and water transport and the subsequent re-adjustment of the turgor pressure still need to be demonstrated. Additionally, due to the fact that the interaction between the CTAD of the PM H^+ ATPase and 14-3-3 proteins requires a change in the phophorylation status of probably both proteins several kinase- and phosphatase-including signal transduction pathways may be involved, e.g. a MAP kinase kinase is activated upon hydration of tobacco pollen (Voronin et al., 2004). Although the knowledge on osmoregulation in plant cells is still fragmentary, pollen grains may serve as a suitable model system for investigating plant osmoregulation and search for an osmosensor.

Materials and Methods

Plant material. *Lilium longiflorum* Thunb. plants were grown in a green house under environmental light and temperature conditions. Pollen grains were collected from fully developed flowers and either used immediately or frozen as single anthers (turgor pressure measurements and pH measurements) or in aliquots of 25 flowers (biochemical experiments) in liquid N_2 and stored at -80°C. In parallel to or before the experiments, aliquots of the frozen pollen grain batches were tested for their germination ability. In all experiments shown, no differences in germination frequency or tube morphology were observed between the frozen and fresh pollen.

Combined turgor pressure and membrane potential measurements. The turgor pressure of ungerminated pollen grains was measured with the pressure probe (Zimmermann et al., 1969) as described in Benkert et al. (1997). Ungerminated pollen grains were suspended in standard bath medium (Med M, in mM: 298 mannitol, 1 KCl, 0.1 Ca(OH)$_2$, 1.6 H_3BO_3, 25 MES (2-morpholino-ethanesulfonic acid) adjusted to pH 5.6

with Tris (Tris[hydroxymethyl]amino-methane), ca. 320 mosmol kg^{-1}) and transferred to a perfusion chamber. A pollen grain was impaled with the oil-filled micropipette of the pressure probe while gently holding the pollen grain at the bottom of the chamber with a second pipette. After successful impalement the holding pipette was carefully withdrawn and the impaled pollen grain was lifted from the chamber bottom. The chamber was constantly perfused with bath solutions of different osmolalities adjusted with mannitol (190 – 500 mosmol kg^{-1}). The turgor pressure was recorded with a chart recorder (Norma Goerz Instruments, Wiener Neudorf, Austria) or digitized at a sampling frequency of 100 Hz and filtered at 10 Hz cut-off frequency using the Digidata 1200 A/D converter and the Axoscope software (Molecular Devices, Sunnyvale, CA, USA). During some turgor pressure measurements pressure pulses were applied to calculate the hydraulic conductivity (L_p) from the subsequent pressure relaxation curves or the volumetric elastic modulus (ε) from small pressure increments (Boyer, 1995; Steudle, 1993).

For simultaneous recordings of turgor pressure and membrane potential a new pressure probe was constructed according to Zhu ((1996), Fig. 2A). Micropipettes were pulled from glass capillaries containing a glass filament (GC 120F-10, Science Products, Hofheim, Germany) with a horizontal pipette puller (Sachs-Flaming PC-84, Sutter Instruments, Novato, CA, USA). The tip of the micropipettes was filled with silicon oil (AS-4, Wacker Chemie, Burghausen, Germany) and back-filled with electrolyte (90 mM K^+ acetate, pH 7.3, 10 mM KCl, see Fig. 2A). When mounting the filled micropipette into the pressure probe holder, an Ag/AgCl-wire reaching into the electrolyte solution was connected to a voltage amplifier (Y-Science, Glasgow, UK). The electrolyte solution was drawn to the micropipette tip due to the capillary forces between the glass filament and the pipette wall. The electric connection was tested after the micropipette and the reference electrode (1 M KCl) were submersed into the standard bath medium. Pollen grains were impaled as described above and the membrane potential difference (V_M) and the turgor pressure were recorded with a two-channel chart recorder.

External pH measurements. Mature pollen grains of one anther were resuspended in an 'unbuffered' medium (Med M) containing (in mM): 298 mannitol, 1 KCl, 0.1 $CaCl_2$, 1.6 H_3BO_3, and the pH was adjusted to 5.6 with tiny amounts of MES or Tris, respectively, if necessary. The pollen grains were washed 3 to 5 times with Med M by short centrifugations to remove the pollenkitt, ions or other compounds bound to the cell wall. Finally, 1 ml of the pollen suspension was added to 1 ml Med M in the multi-port measurement chamber (WPI, Berlin, Germany) housing the reference as well as the pH electrode (Kwik-Tip, WPI). The electrodes were connected to an electrometer (610C, Keithley Instruments, Cleveland, OH, USA) and the data were sampled at 333.3 Hz by an A/D converter (DigiData 1200, Molecular Devices), filtered at 10 Hz cut-off frequency and recorded with the Axoscope 7 software. The quality of the pH electrode was tested before each experiment by measuring solutions with known pH values giving slopes of 50 to 56 mV per pH unit. The pH value was calculated from the recorded voltage using the calibration curves. The acidification (negative values) or alkalinisation rates (positive values) are given as $\Delta pH\ min^{-1}$. During the measurements the PG suspension was constantly stirred at slow speed and osmolality was changed by adding appropriate

volumes of hyper- or hypo-osmotic mannitol stock solutions (with all other components as Med M) to give 198 mM and 398 mM mannitol, respectively, as final concentrations.

Plasma membrane isolation, gel electrophoresis and immunodetection. PGs from 75 flowers were incubated for 15 min in Med M, split to 3 aliquots which each aliquot incubated for another 15 min in iso-osmolar Med M (298 mM mannitol), in hypo-osmolar Med M (198 mM mannitol) and in hyper-osmolar Med M (398 mM mannitol). Fractions of soluble cytosolic (CF), crude membranes (MF) and plasma membrane-enriched (PMF) proteins were prepared from each pollen aliquot by differential centrifugation followed by discontinuous sucrose density gradient centrifugation as described by Pertl et al (2005, 2009).

Proteins were separated by SDS-PAGE by denaturing protein fractions (10 - 20 µg protein) in sample buffer (300 mM DTT, 30% (w/v) SDS, 15% (w/v) glycerol, 180 mM Tris/HCl pH 6.8, bromphenol blue) at RT for 15 min and loading onto a discontinuous gel system (Laemmli, 1970) with 4 % stacking gel and 10 % separation gel (Protean 3 system, Biorad, Vienna, Austria). Proteins were stained with Coomassie Brilliant Blue R-250. The protein concentration was determined using a Lowry DC assay (Biorad).

For immunodetection separated proteins were transferred onto PVDF membranes (Roth, Karlsruhe, Germany) by electro-blotting with 20 V for 1 h (Semi Dry Electrophoretic Transfer Cell, Biorad) and immunodetection of the PM H^+ ATPase and 14-3-3 proteins was performed as described previously ((Pertl et al., 2005)) with the following antibody concentrations and combinations: MUP5 and MUP15 diluted 1:250 and detected by goat anti-mouse IgG AP-conjugated (1:5,000 or 1:10,000, Sigma) for 14-3-3 detection. For immunodetection of the PM H^+ ATPase the primary antibody was diluted 1:2,500 and secondary antibody goat anti-mouse IgG-AP was diluted 1: 5,000 (Sigma).

PM H^+ ATPase activity. Hydrolysis of ATP by the vanadate-sensitive PM H^+ ATPase was monitored by determination of released inorganic phosphate (Ames, 1962) as described in Pertl et al. (2005).

Supplemental Material

Fig. S1 Determination of the hydraulic conductivity (L_P).

Fig. S2 Acidification of the external medium by an active PM H^+ ATPase.

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Figure Legends

Fig. 1 Turgor pressure of pollen grains. A. A lily pollen grain was impaled with the pressure probe in standard medium (298 mM mannitol). Perfusion of the chamber with hyper- (398 mM mannitol) and hypo-osmolar (198 mM mannitol) media resulted in a reversible change of turgor pressure. At the end of the turgor pressure measurement, small pressure changes were induced and the corresponding volume of the pollen grain measured to determine the elastic modulus ε. **B.** The measured turgor pressure plotted against the osmolality of the bath medium (n > 8 individual pollen grains) showing a linear osmotic behavior of lily pollen grains. Mean values ± S.D.

Fig. 2 Simultaneous measurement of turgor pressure and membrane potential in single pollen grains. A. Modified pressure probe with a chlorided silver wire reaching into the electrolyte solution. Except for the indicated part of the micropipette, the pressure probe as well as the other parts of the micropipette was filled with silicon oil. **B.** Typical recording of the turgor pressure (black) and membrane potential V_M (gray). A change from iso- to hypo-osmolar medium (198 mM mannitol) increased the turgor pressure and depolarized the plasma membrane whereas (**C.**) a change to hyper-osmolar medium (398 mM mannitol) led to a hyperpolarization of the plasma membrane. Stars indicate the time at which the data were used to create Fig. 2D. **D.** Summary of all pollen grains in which turgor pressure and V_M were measured simultaneously. Hyperpolarization of the plasma membrane upon hyper-osmolar treatments are given as negative ΔV_M and Δosmolality values, respectively. A regression line with the 95% confidence intervals (dashed line) was fitted to the data.

Fig. 3 Vanadate-sensitive H^+ transport changes upon hypo- and hyper-osmotic shock. A. Pollen grains were incubated in standard medium (298 mM mannitol) and the pH of the external medium was monitored. Upon sudden osmotic changes (198, 298, and 398 mM mannitol) at t = 0 min, temporal changes in the pH values can be noticed, namely an alkalinisation after hypo-osmotic shock, no changes after iso-osmotic additions and an increased acidification rate after hyper-osmotic challenge. The first 100 s were fitted with a linear regression line giving an pH change rate of 0.0081, 0.0010 and -0.0180 pH units min^{-1} for 198, 298 and 398 mM mannitol treatments, respectively. **B.** The low acidification rate before the osmotic challenge

was sensitive to vanadate and acidification rates did not change after addition of iso-osmolar medium. The alkalinisation in the first 100 s after hypo-osmotic and the increased acidification rate after hyper-osmotic challenge were inhibited by the presence of 500 µM vanadate. Mean values ± S.D. (n ≥ 3).

Fig.4 PM H$^+$ ATPase and membrane-associated 14-3-3 proteins after osmotic treatment. Pollen grains were incubated in standard medium (298 mM mannitol) for 15 min and exposed to different media osmolalities (198, 298, 398 mM mannitol) for another 15 min. The cytosolic (CF), membrane (MF) and plasma membrane-enriched fractions (PMF) for each treatment were isolated. Proteins were separated by gel electrophoresis, blotted onto PVDF membranes and detected by specific antibodies as indicated. **A.** No changes in signal intensities of the PM H$^+$ ATPase in the MF and of 14-3-3 proteins in the CF are observed, whereas 14-3-3 proteins in the MF showed a signal change dependent on the osmotic pre-treatment. 10 µg protein per lane. **B.** Compared to iso-osmolar treatment (298 mM), higher 14-3-3 signals after hyper-osmotic shock (398 mM) and lower 14-3-3 signals after hypo-osmotic shock (198 mM) can be observed in the PMF with 2 different antibodies against 14-3-3 proteins. The amount of PM H$^+$ ATPase was not affected by osmotic treatments. 20 µg protein per lane **C.** Coomassie-stained gel of fractions used in A and B for immonudection to verify the loading of 10 µg protein per lane (loading control).

Tables

Tab. 1 PM H$^+$ ATPase activity. The vanadate-sensitive ATP hydrolysis was measured in plasma membrane-enriched fractions prepared from PGs incubated in hypo (198 mM), iso (298 mM) and hyper-osmolar (398 mM mannitol) germination medium. The specific activity is given in nmol mg^{-1} min^{-1}. Mean values ± S.D. (n = 3).

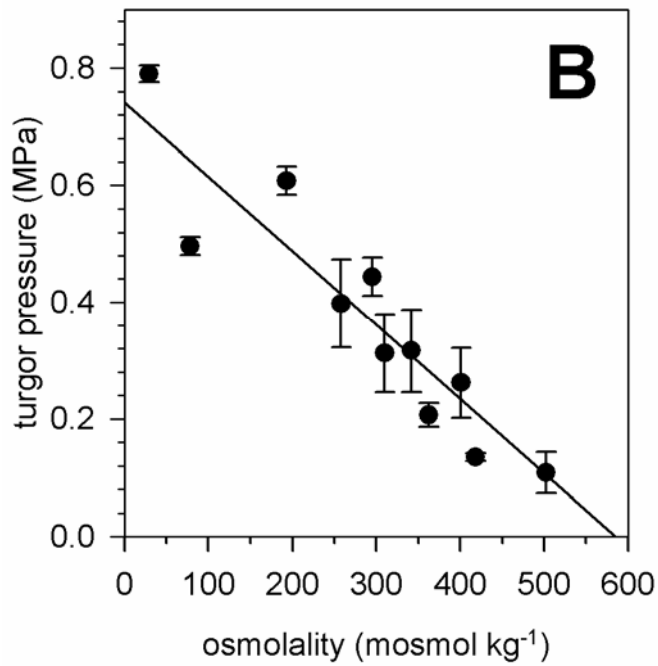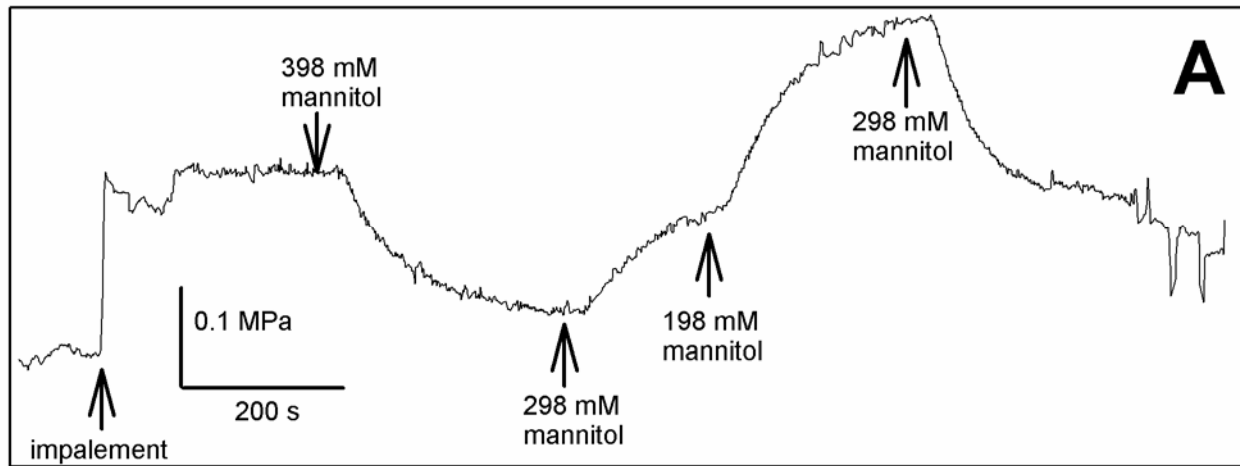| mannitol conc. (mM)	specific activity nmol P$_i$ mg^{-1} min^{-1}	
198	89.50 ± 19.92	hypo
298	130.20 ± 7.35	iso
398	164.29 ± 30.30	hyper

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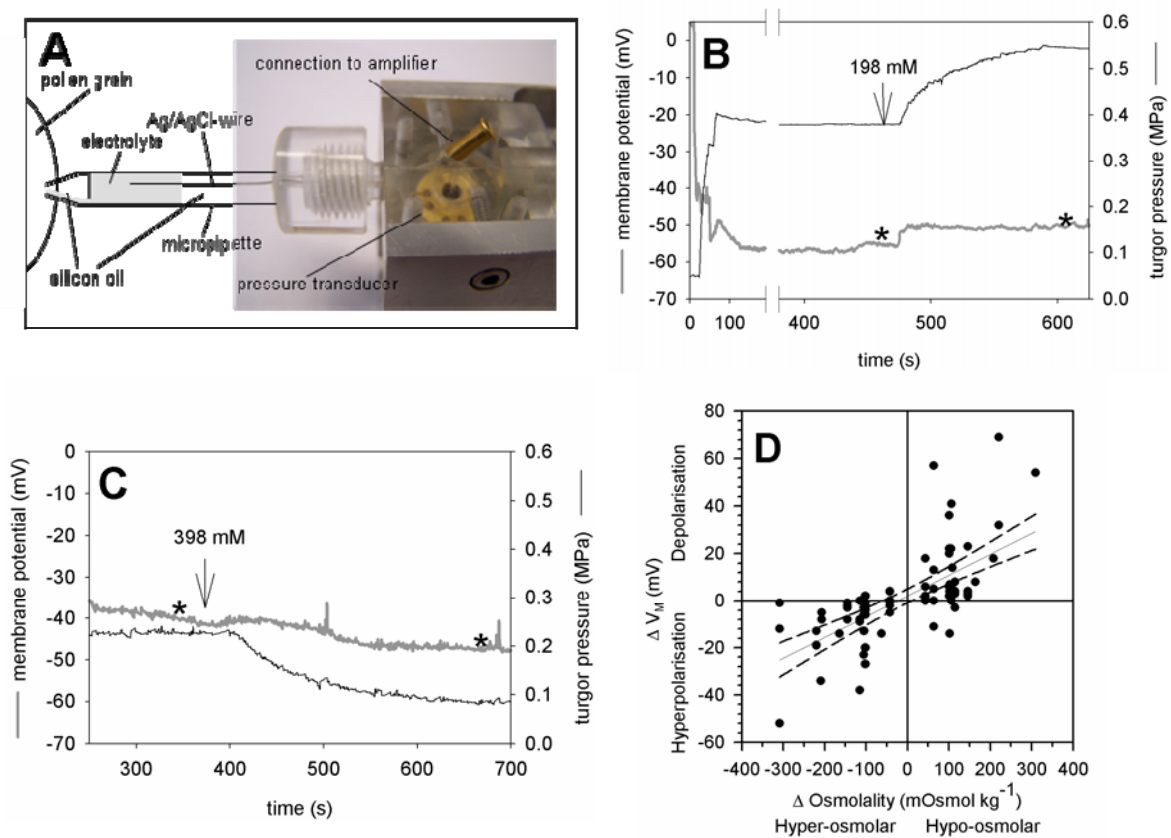
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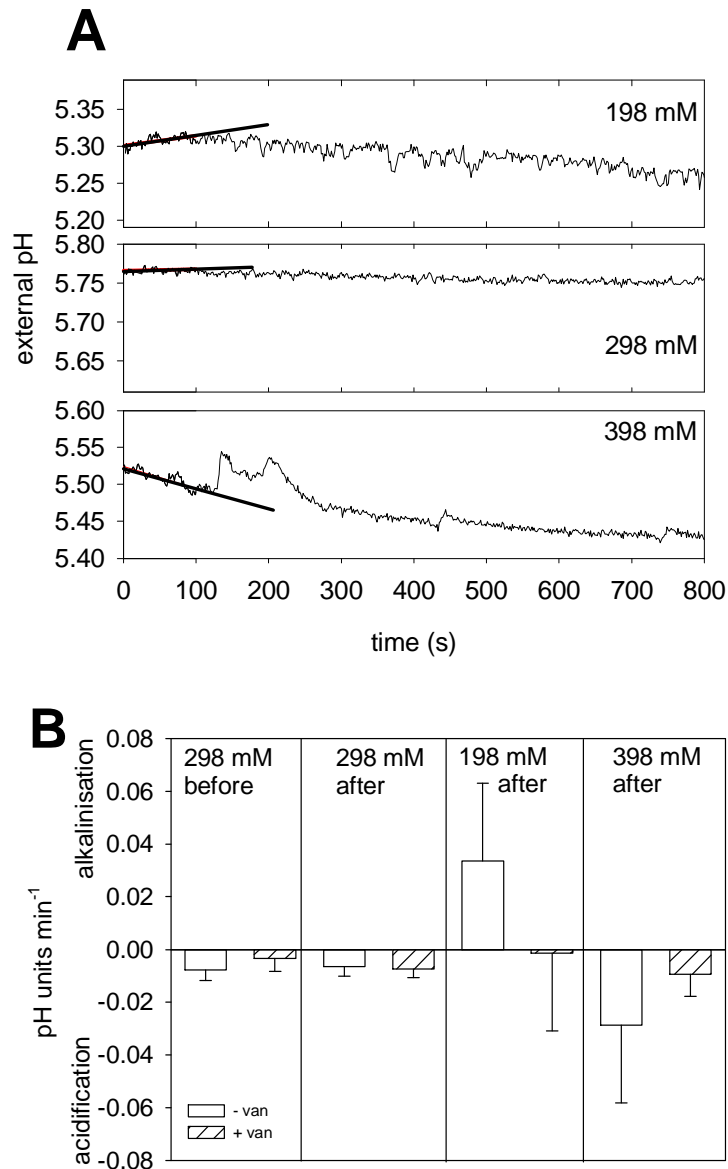
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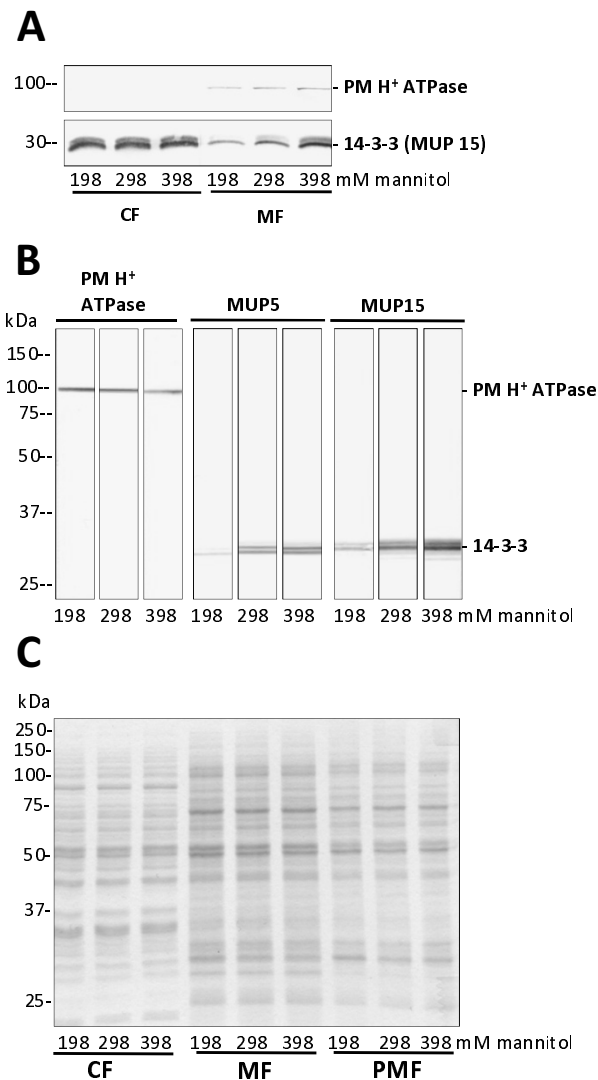
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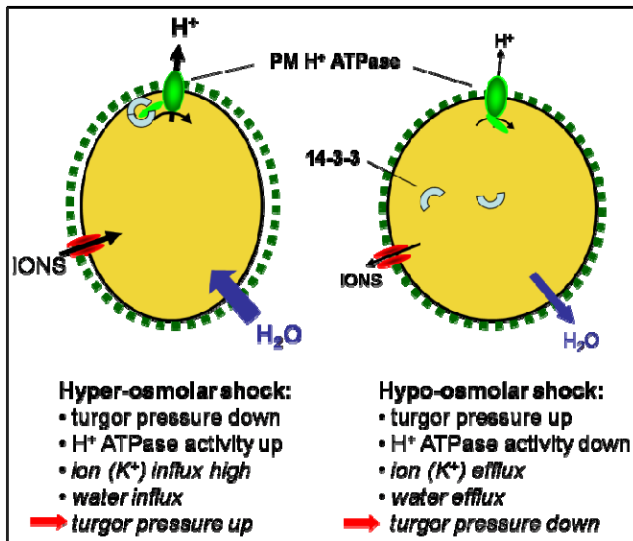
Fig. 5 Schematic summary of osmoregulation in lily pollen grains. The first two events after osmotic shock were measured in this study. Subsequent, hypothetical events finally leading to a re-adjustment of turgor pressure are given in italics. For further explanation see Conclusion.

Fig. 5