The role of ion fluxes in polarized cell growth and morphogenesis: the pollen tube as an experimental paradigm

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ABSTRACT In order to cope with reproduction in a dry environment without any sort of motility, plants have developed a very specialized and unique sexual system. Of special notice, the two sperm cells that will perform the double fertilization typical of higher plants are carried by one of the fastest growing cells in nature, the pollen tube. This tube develops from the vegetative cell of the pollen grain upon germination on the female tissues. While it cannot be considered as a canonical excitable cell, pollen tubes depend for most of their fundamental functional features on a close regulation of ion dynamics, namely in terms of polarization of extracellular fluxes and formation of standing cytosolic free ion gradients, namely of calcium (Ca\(^{2+}\)) and protons (H\(^+\)). In turn, these imply that plasma membrane transporters are polarized, or polarly regulated, and that internal signaling cascades transduce this spatial information into the basic features of growth and morphogenesis needed for pollen tubes to target correctly the ovules and discharge the sperm cells. Because of the singularity of this organization, and the ease with which pollen tubes can be experimentally handled, recent years have witnessed an accumulation of data at many levels, from basic biophysical and cell biology characterization, to gene assignment and transcriptomic description of pollen development. In this review we aim to organize this information in terms of the basic biophysical features of membrane function and integrate it into conceptual testable hypotheses on how the dynamics of ion regulation may underlie fundamental properties of cell development.

KEY WORDS: Plant development, pollen, ion channel, proton pump, cell polarization

Introduction

Evolution has shaped higher plants with an extraordinarily specialized and differentiated sexual reproduction system that allowed the adaptation to sessile habits and dry environments, in turn making it possible for plants to successfully colonize all habitats. Among these evolutionary singularities, stems the capacity to transport and deliver non-motile male sperm from great distances apart. The system basically works through the development of the pollen grain, the male gametophyte of plants. This organ is released to the atmosphere in a highly dehydrated state, surrounded by a "bullet-proof" external wall. These features make pollen fit to endure large trips through a possibly aggressive atmosphere, until landing on the female sexual organ, the pistil. If the cross is compatible and viable, re-hydration of pollen takes place and germination occurs, giving rise to the pollen tube (Boavida et al., 2005a,b).

The pollen tube is a highly specialized cell that results from the germination of pollen and the outgrowth of its vegetative cell after pollination. This fast growing cytoplasmic extension (or tube) serves to transport the sperm cells contained within the pollen grain from the external surface of the female reproductive organs (the stigma) down to the core of the ovary, and eventually deliver them to the ovule for the typical double fertilization of plants. This implies a long and very rapid, divisionless, growth of the pollen tube (reviewed in Boavida et al., 2005a,b). In fact these cells line-up within the ones that grow faster in nature, with linear growth rates up to 4 \(\mu m sec^{-1}\) (e.g. in species like Tradescantia or Hemerocallis) and extension lengths up to 40-50 cm (e.g. maize). Having in mind that a typical pollen grain is not much more than

Abbreviations used in this paper: Em, electrical membrane potential; Nt AHA, nicotiana H\(^+\) ATPase; PM, plasma membrane.
a 100 μm spheroid organ, this may imply a multiplication of volume of several orders of magnitude, sometimes within a few hours.

To sustain this amount of growth, pollen tubes must have by default an efficient ion transport system at the plasma membrane (PM). Indeed, the volume increase of the tube induced by its extension implies water and solute influx into the cell and accumulation into its expanding vacuole. On the other hand, the growing tube accumulates new charged organic material, which implies the corresponding entry of specific ions to compensate the charge balance of the newly acquired or synthesized molecules.

More interestingly, in addition to playing a role in the growth itself, ion transporters and other interacting proteins make certain ions pivotal components of the cell signaling network by themselves. Overwhelming amounts of literature prove that to be the case for calcium (Ca\(^{2+}\)) but significant evidence shows that to be the case for others as well, most notably protons (H\(^{+}\)). These transduction pathways are presumably involved in the self-organization of pollen after re-hydration, and therefore are expected to have roles in the control of polarized establishment of growth that eventually leads to the differentiation and sustained growth of the pollen tube (Feijó \textit{et al}., 1995; 2001; 2004; Boavida \textit{et al}., 2005b).

Pollen tubes also share many commonalities with a number of other apical growing cells, namely root hairs, fungal hyphae or in certain cases neurite outgrowths (see e.g. Palanivelu and Preuss, 2000). Of special relevance for comparative purposes, root hairs play a central role in the high affinity uptake of nutrients by the root. It becomes therefore natural that these two cells have been elected as prominent plant cell models for the study of polarized growth. In addition to common cellular features at the level of shape and cytoplasm polarization, root hairs also developed unique ion dynamics systems for signaling (see e.g. Cárdenas \textit{et al}., 2000).

Many of the central issues arising from the studies of pollen tubes and root hairs have been defined around cell shape and sub-cellular polarization. The study of ion transporter systems is a key point to address both of these questions, because to date all fluxes, intracellular free concentration, and sub-cellular localization of transporters were shown to be conspicuously polarized. Substantial evidence also showed that ion transporters are necessary for cell organization, presumably through the control of polarization via signaling networks. Last but not least, ion transporters play important roles in the respective organ physiology, namely allowing a fast growth and rapid uptake of ions, presumably underlying an efficient high affinity uptake of molecules.

Recently, high-throughput genetic data was obtained in the pollen grain, with the description of the nearly-full transcriptome of Arabidopsis pollen (Becker \textit{et al}., 2003; Honys and Twell, 2003, 2004; Pina \textit{et al}., 2005). In parallel, substantial efforts are also being made on the pollen proteome (e.g. Noir \textit{et al}., 2005), opening new possibilities to establish the pollen tube as a model system (see review by Becker and Feijó, 2007). In short, the wealth of molecular data published in the recent years allowed a better understanding of the pollen tube ion transport system and set the background for specific hypotheses to be tested in terms of functional genetics.

In this review, we will describe the molecular nature and the role of ion transport systems in the pollen tube physiology in the light of these new results. Furthermore, we will discuss the implications and shortcuts of current models for the role of the ion transport polarization in nutrient uptake efficiency.

### Pollen tubes: an electrically exciting cell!

Ever since the pioneer studies in the group of Lionel Jaffe established cells as generators of physically well defined electrical currents, pollen tubes are known to behave as electrical dipoles, with outward (positive charges) currents arising from the grain, and inward currents leaking the apical parts of the tube (Fig. 1A; Weisenseel \textit{et al}., 1975; Weisenseel and Jaffe, 1976). Most of this data was acquired with a then newly developed method, the vibrating-probe, which consisted of a metal electrode measuring voltage differences between two adjacent points, thus inferring the total electric current from the difference between the sum of charge activity in the two points. While establishing a whole new field in terms of physiological development, this original version of the vibrating probe was also posed with technical limitations that hindered some of the biologically relevant properties inherent to these currents. Most notably, these probes effectively agitated the medium at high frequencies, thus destroying small or slow moving ion gradients generated around cells. Moreover, the determination of the specific ion species underlying the current was not possible. Some approaches were attempted with substitution/addition experiments, but a combination of technical issues made it hard to interpret the data in terms of the specific ions that carry those currents.

A few groups concurred then to develop ion-specific vibrating probes, today known under different acronyms, but basically all exploring the same sort of biophysical properties of artificial ionophore cocktails (e.g. SIET, SERIS, MIFE, see Kunkel \textit{et al}., 2006).

Application of these methods to pollen tubes soon allowed the drawing of a map of the main inorganic ion fluxes they drive. Amazingly, most ion fluxes seem to follow what could be characterized as a “short-circuit” loop across the pollen tube (see Fig. 1B,C). In sheer thermodynamic terms, this scheme implies an obvious waste of energy, and therefore a justification for these “short-circuits” must be sought in terms of local “energization” for tighter, spatially restricted control.

Ca\(^{2+}\) and H\(^{+}\) have been reported to enter massively the tip (Feijó \textit{et al}., 1999; Feijó, 1999; Messerli \textit{et al}., 1999; Robinson and Messerli, 2002). Protons then leak out mostly through the PM of the tube and the grain (Feijó \textit{et al}., 1999; Feijó, 1999; Certal \textit{et al}., 2008). Anions, most notably chloride (Cl\(^{-}\)) have been shown to have a reverse direction, entering the tube / grain and leaving massively through the tip (Zonia \textit{et al}., 2002). This first work was performed using a low pH buffer concentration (MES in the μM range), a condition necessary to optimize signal/noise (S/N) ratio when using artificial ion probes. A different set of conditions was used by Messerli \textit{et al}., 2004, and under low buffer concentrations the authors obtained the same results as Zonia \textit{et al}., 2002. Messerli \textit{et al}., 2004 however attempted to create a number of other experimental conditions to dismiss a role for Cl\(^{-}\) in pollen tube, but to our eyes, these authors not only failed to reproduce our exact measuring conditions, as they failed to build a sufficiently compelling case to rule out a role for Cl\(^{-}\) in pollen tube regulation. Of special notice, they chose to ignore a number of other pieces of evidence using pharmacology, specific inositol...
Role of ion fluxes on pollen tube growth

Fig. 1. Current model for ion fluxes around the pollen tube: an active transport system at the tube, and a passive transport system at the tip. (A) Electrical model for the lily (Lilium longiflorum) pollen tube, deduced from the extracellular electric currents, as determined by the original voltage-vibrating probe. A net current of positive charges enters the tip, leaking by the tube and grain, is hypothesized to transverse the cytoplasm with a net intensity of about 1 pAmp (adapted from Weisenseel et al., 1975). (B) Inorganic ion fluxes around the pollen tube of tobacco (Nicotiana tabacum) as recorded using the ion-specific vibrating probe technique. Ca\(^{2+}\) and H\(^{+}\) leave the pollen tube, via ATPases and enter the tip, via channels for Ca\(^{2+}\), and non-identified transporters for H\(^{+}\). Cl\(^{-}\) enters the tube via non-identified transporters and leaks from the tip via channels. Data for K\(^{+}\) is still controversial. (C) Electric equivalent of the hypothesized polarization of transporters in the tobacco pollen tube. (D) Analysis of transcriptome for ion transporters in the pollen of Arabidopsis. Dark grey areas represent the total number of genes categorized for each family of transporters in the Arabidopsis genome. Small squares represent the ones present in pollen, colored from red (over-expressed in relation to the vegetative tissues) to blue (under-expressed). (data from Pina et al., 2005; see also Becker and Feijó, 2007).

Curiously enough, the same group described strong influxes of potassium (K\(^{+}\)) at the tip, but mentioning no other coupled fluxes...
able to drive and balance these charge fluxes (Messerli et al., 1999). Although our group intensively worked on K⁺ fluxes around pollen tubes, the reproducibility of these tip influxes remains challenging. It is noteworthy that the experiments published were performed using the ion-vibrating-probe technique, with very high buffer (MES 5 mM), but more importantly, high K⁺ backgrounds (possibly over 3 mM). The incorporation of large quantities of buffer in the bath medium has been simulated and experimentally confirmed to produce large artifacts using ion-vibrating probes (Kunkel et al., 2001). In all likelihoods the same sort of artifacts are theoretically expected for measures on high K⁺ backgrounds. In these conditions, changes of the measured electric potentials with the probe in relation with K⁺ concentration in the media are expected to be very small, and with significantly low S/N ratios, turning these results difficult to interpret. It is curious to note that the group of Robinson demonstrated that the experimental conditions used to measure these K⁺ fluxes – i.e. high MES concentration – do not necessarily fit with a precise measurement using the ion-specific vibrating probe technique, an argument used to dismiss a role for Cl⁻ in pollen tube growth (Messerli et al., 2004).

The possible consensus of all this information is gathered in Fig. 1B, and the equivalent electric diagram in Fig. 1C. So how do all these fluxes cope with the original finding that pollen tubes behave as an electric dipole? The charge movement across the pollen tube must be carried by the ions fluxes described above. Nevertheless, only a fraction of these ion fluxes is involved in the net charge movement across the pollen PM. For instance, in the PM of the tube, charge effluxes driven by the proton-pump may be partially compensated by cationic influxes, such as K⁺. Furthermore, being an open system, pollen tubes have net accumulations of certain ions and net extrusion of others, a notion which stems as almost intuitive from the fact that ion fluxes at the tip and at the tube are both qualitatively and quantitatively different. Consequently, the net charge flux at the tube can be driven by ions different than the ions driving the net charge influx at the tip (Fig. 1 A-C). Potassium substitution and addition experiments have lead to previous conclusions that K⁺ influx at the tip could be responsible for the net charge influx (Weisenseel and Jaffe, 1976). Besides the issues related to the use of the original voltage vibrating probe to infer ion specificity, the data discussed in the previous paragraphs, namely the presence of proton influxes and strong anion effluxes at the tip, make this model no longer satisfactory. Any accurate description of the specific mechanisms underlying the differential polarization of fluxes must have into consideration what is nowadays known about the specific ion transporters expressed in pollen for each of the involved ions (Fig. 1D). This is a challenge that should be interiorized by every group in the field if one aims to escape the vicious circle of continuous contention based on qualitative results, basically obtained through different experimental conditions based on personal or historic instrumental interpretations and consequently, independent qualitative subjective reading of the data in terms of the underlying molecular mechanisms.

**Ion transporters and cell growth**

Ion transporters are both involved in signaling, via Ca²⁺ and H⁺ or membrane potential (Eₘ), and in cell growth, by permitting the accumulation of ions inside the cell, and/or promoting turgor pressure. Therefore, they are an intrinsic part of the growth machinery while playing a central role in triggering different signaling cascades. Remarkably, ion transporters must achieve these multi-functional roles by self-organization of the existing proteins in the pollen grain before dehydration and release into the atmosphere, or by the ones immediately expressed after germination, thus possibly creating an original paradigm for self-organization of dehydrated germinating cells (Feijó et al., 1995). It might be noted that ion transporters act in systems, and that the activity of all the ion transporters in one system are inter-dependent. As described in the following section, ion transporters...
activity affects many biophysical parameters, which in turn could all take part in the fundamental features underlying cell growth and signaling. But first one must describe their known effects on the intracellular milieu.

**Cytoplasmic polarization and second messengers**

Many studies showed that calcium displays a concentration gradient at the tip of the pollen tube, namely by using radio-labeled calcium (Jaffe et al., 1975) or synthetic fluorescent probes (Reiss and Herth, 1978; 1979; 1985). These methods were later refined to demonstrate the presence of a tip-focused cytosolic free Ca\(^{2+}\) gradient, a much more important finding for issues of signaling than total cytoplasmic Ca\(^{2+}\) (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Malhó et al., 1994; Pierson et al., 1994; 1996; Messerli and Robinson, 1997; Holdaway-Clarke et al., 1997; Messerli et al., 2000). More recently, genetic probes were introduced, confirming this pattern (Watahiki et al., 2004; Iwano et al., 2004; see Fig. 2). The precise role of the extracellular Ca\(^{2+}\) influx at the tip in the Ca\(^{2+}\) gradient establishment is still unclear (Holdaway-Clarke et al., 1997; Holdaway-Clarke and Hepler, 2003). Treatment of pollen tubes with Ca\(^{2+}\) channel inhibitors disrupts the Ca\(^{2+}\) gradient, demonstrating that the Ca\(^{2+}\) gradient is apparently triggered by its influx (Obermeyer and Weisenseel, 1991; Malhó et al., 1995). Nevertheless, a role for Ca\(^{2+}\) internal stores was also proposed as an alternative explanation for the gradient establishment (Holdaway-Clarke et al., 1997). In summary, the Ca\(^{2+}\) gradient at the tip seems to be essential for the pollen tube to grow, and in addition to act as modulator of the directional changes of growth, with some evidence showing a possible causal role on the re-directioning response, as the delocalization of this gradient precedes pollen tube turning (Malhó and Trewavas, 1996).

Cytoskeleton dynamics / exocytosis (Roy et al., 1999; Parton et al., 2003; Hwang et al., 2005), endocytosis (Helling et al., 2006) and ion transporters (Becker et al., 2004) have been proposed as the physiological effectors of the Ca\(^{2+}\) gradient. The signaling cascades downstream Ca\(^{2+}\) are multiple (Malhó et al., 2006), and may imply phosphorylation through Ca\(^{2+}\)-dependent-protein kinases (Yoon et al., 2006), small GTPases (Gu et al., 2005) or calmodulin (Rato et al., 2004). The general scheme of the Ca\(^{2+}\)-dependent signaling cascades is largely unknown in pollen, despite the obvious intercalation and ramification of the various networks (see e.g., Holdaway-Clarke and Hepler, 2003).

In parallel, H\(^+\) enters the tip, but leaves steadily through tube shank and grain PM (Fig. 1B). Imaging studies revealed a H\(^+\) gradient inside the pollen tube, with an acidic end located at the tip, and an alkaline zone grossly corresponding to the clear zone of large organelles in lily (Fig. 4A; Feijó et al., 1999). The H\(^+\) influx at the tip has been suggested to be partly responsible for the establishment of this gradient, which in turn may be responsible for some of the features of the actin cytoskeleton in that area (Cárdenas et al., 2005; Lovy-Wheeler et al., 2006). A role for internal H\(^+\) stores (mitochondria, vacuole and secretory vesicles notably) is also expected for the maintenance of this gradient (Feijó et al., 1999). Most importantly, the specific location on the plasma membrane of the H\(^+\)-pumps is predicted to be of pivotal importance for the dynamic regulation of this gradient (Feijó et al., 1999; 2004; Certal et al., 2008).

Being the two best cytosolic free ions studied so far, one may speculate about the synergistic relationships between Ca\(^{2+}\) and H\(^+\)? The presence of both H\(^+\) and Ca\(^{2+}\) standing concentration gradients is a sine qua non condition for pollen tubes to grow, and both have sometimes overlapping functional targets in the cell. Of special note is actin dynamics and transporters, an aspect argued to defend the point of a synergistic role between both ions (Lovy-Wheeler et al., 2006). The imaging of cytosolic [Ca\(^{2+}\)] and [H\(^+\)] using genetic markers shows that the two gradients display different spatial patterns. In tobacco, the Ca\(^{2+}\) gradient displays a cortically focused shape, slightly de-focused from the tip (Fig. 2), while the H\(^+\) gradient shows up as an inverted-cone (Fig. 3)(Michard et al., 2008). Of special notice, the [Ca\(^{2+}\)] imaging was done using particularly stringent confocal imaging. We contend that this differential spatial patterning of these two second messengers may play a role in the pollen tube polarization and spatial organization of the tip domain.

**Does a hydrostatic field operate at the pollen tube?**

There is no correlation between the absolute turgor pressure and pollen growth rate, but a minimum intracellular turgor pressure is required for pollen tube growth (Benkert et al., 1997). The role of turgor pressure in the mechanical driving of the pollen growth is therefore ill-defined. It is noteworthy that the measured turgor pressure is substantially inferior to the external osmotic pressure needed to plasmolise the cell (Benkert et al., 1997). This can be a clue that other hydrostatic forces take place inside the pollen tube.

Despite the fact that no experimental confirmation was yet published, it has been contended that the pollen tube displays a unique volume of vacuole-free cytoplasm (Mascarenhas, 1993). This cytoplasmic fraction is by and large devoid of any large organelle thereby being able to support water-potential differentials. In this space, local hydrostatic forces can not be neglected, such as the ones that derive from hydrogel interactions of macromolecules, namely globular proteins, with water. Note also that anions massively leave the tip. Predictably this increases the water-potential inside the cytoplasm near the apical PM (Zonia et al., 2001, 2002). The large volume of the cytoplasm between the tip PM and the vacuole may then allow the establishment of a water-potential gradient inside the cytoplasm and the associated hydrostatic forces. These mechanical forces are expected to be directed from the tube in the apical direction and may modulate locally the mechanical pressure at the tip PM.

**The molecular identity of ion transporters in the plasma membrane of pollen tubes**

The proton-pump is restricted to the basal plasma membrane regions and energizes the plasma membrane

Pollen germination is dependent on the activity of H\(^+\)-pumping, a finding based on various observations, namely on the general correlation between activators and inhibitors of these pumps and germination rates. One of the first reports was in olive, where germination rates correlated with H\(^+\) extrusion, as measured by the medium acidification, either by stimulating with fusicoccin or inhibiting with vanadate (Rodríguez-Rosales et al., 1989). In various other species, experimental and genetic conditions seem to corroborate this early finding (Feijó et al., 1992; Zhao et al., 2000; Robertson et al., 2004; Lovy-Wheeler et al., 2006; Certal et al., 2008)
Direct demonstration of a H⁺-pump activity, agonized by fusicoccin and inhibited by vanadate, has been made by patch-clamping pollen tube protoplasts (Gehwolf et al., 2002). Enzyme cytochemistry and immunochemistry were also used to reveal the differential presence of an ATPase activity in the pollen grain and tube PM (Feijó et al., 1992; Obermeyer et al., 1992; see Fig. 4E). Generally speaking, the PM of pollen tubes displays a net efflux of H⁺ as measured using a proton-specific vibrating probe both in lily (Fig. 4B; Feijó et al., 1999) and tobacco (Fig. 4F; Certal et al., 2008). In Lilium, the H⁺-efflux has a maximum intensity at around 50 μm far from the tip (Feijó et al., 1999). This H⁺ efflux peak corresponds to the location of an alkaline domain in the cytoplasm, as measured using the fluorescent pH-probe BCECF-10.000 Dextran (Fig. 4A; Feijó et al., 1999). This suggests that H⁺-pumps are active on the tube PM and deplete the cytoplasm of H⁺. Confirming the assumption, treatment by fusicoccin (that activates the H⁺-pump) alkalinizes the pollen tube cytoplasm (Lovy-Wheeler et al., 2006). A theoretical prediction that could explain these results would be the specific exclusion of these pumps from the apical PM (Fig. 4C; Feijó et al., 1999, 2004).

Various molecular studies confirmed the presence of H⁺-pumps and ultimately the prediction that they are absent from the apical domain of the PM. Arabidopsis pollen expresses several isoforms of the H⁺-pumps (the AHA gene family) that could account for this ATPase activity, some being pollen specific and/or highly expressed, most notably AHA6 (Pina et al., 2005). Lefebvre et al. (2005) cloned it and showed by immuno-localization that its putative orthologue in Nicotiana plumbaginifolia, Npma5, is specific of pollen, and absent from the tip. More recently, we cloned another orthologue from tobacco, which was baptized as Nt AHA (Nicotiana H⁺-ATPase; Certal et al., 2008). The sub-cellular localization of Nt AHA was studied by GFP fusion, and shown to be, even on over-expressing conditions, by and large absent from the apical PM (Fig. 4D).

**The proton-pump energizes the plasma membrane of the pollen tube**

The electric membrane potential (Eₘ) has been measured in pollen tubes, by impaling an electrode 50 to 100 μm far away from the tip in lily (Weisenseel and Wenisch, 1980) and Arabidopsis (Mouline et al., 2002). Although the method underestimates the measured potential (Gassmann and Schroeder 1994), the values reported are slightly below the equilibrium potential for K⁺, suggesting the presence of an active transporter on the tube PM. Moreover, tube Eₘ is sensitive to external pH, suggesting a role for H⁺ fluxes in its establishment, as well as temperature, suggesting the presence of an active transport system (Weisenseel and Wenisch, 1980). All together, these data strongly suggest that some H⁺-pump system energizes the tube PM.

The role of the Ca²⁺-ATPase in membrane energization has not been considered so far. Yet, genetic evidence showed a function for one such pump, proven to be necessary for pollen development and growth, and expressed evenly on the tube PM, including the apical domain (Schiott et al., 2004). A weak Ca²⁺ efflux has been recorded at the tube shank, but the role of Ca²⁺

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**Fig. 3. Cytosolic pH and H⁺ fluxes at the tip of tobacco pollen tube.**

(A) Intracellular pH of a tobacco pollen tube as imaged with the GFP-derived pH-sensor “pHluorin” (Miesenbock et al., 1998). The image was obtained by ratiometric widefield microscopy, and depicts a typical initiation of a growing cycle, corresponding to a massive influx of H⁺ at the tip, forming a comet-like gradient of acidity. Note however that the flanks of this gradient are significantly more alkaline than the core (arrows), revealing that the sub-membranar volume is likely always less concentrated in H⁺ (i.e. relatively more alkaline) as compared to the tube core (Michard et al., 2008). (B) Cytosolic pH fluctuations (line) and growth rate (bars) of a tobacco pollen tube. The dynamic behavior is qualitatively similar to the one of Ca²⁺, but with slightly different delays and oscillation properties. (C) H⁺ influxes recorded at the tip of tobacco pollen tube using a H⁺-specific vibrating probe.
efflux in PM polarization is expected to be marginal. Moreover, electrical membrane potential (Em) is poorly dependent on external Ca²⁺ concentration (Weisenseel and Wenisch, 1980). The mechanistic implications of this pump remain thus to be better established, but its importance is well spelled out on the consequences of its genetic manipulation.

**Proton pump-dependent ion absorption by the tube**

Being above the equilibrium-potential for K⁺, the tube Em can, per se, implicate the passive entry of this cation. Supporting this idea, the membrane potential of the tube is strongly dependent on external K⁺ (Weisenseel and Wenisch, 1980). Using a pipette impalement technique in voltage-clamp mode, a large conductance for K⁺ was also described across the tube PM (Becker et al., 2004).

Pollen tubes can grow under extremely low external K⁺ concentrations, the only consequence being a slightly slower growth rate. Under low K⁺ growth conditions they become very sensitive to K⁺ channel inhibitors (Fan et al., 2001; Moulène et al., 2002). This suggests that these cells possess a powerful K⁺ uptake system, in which K⁺ channels may play a significant role.

The transcription of several K⁺-channels has been identified in pollen using RT-PCR (Moulène et al., 2002; Becker et al., 2004) or micro-arrays analysis (Becker et al., 2003; Honys and Twell, 2004; Pina et al., 2005). Using pharmacological studies coupled to genetic and electrophysiological approaches, a K⁺ channel from the *Shaker* family (SPIK) has been shown to play a role in this high affinity K⁺ uptake in *Arabidopsis* pollen (Moulène et al., 2002), specially on hyper-polarization conditions. Another member of the *Shaker* family, AKT5, is also expressed in pollen (Becker et al., 2004). The functional properties of AKT5 are unknown. Other members from the family may also play a role (Moulène et al., 2002; Becker et al., 2004). More recently, a K⁺ channel, AtTKP4, from the KCO/TPK family was shown to be responsible for a marginal proportion of the tube K⁺ conductance (Becker et al., 2004). Other transporters, from CHX or AKT/ AKUP/HAK families for instance, may contribute to the K⁺ fluxes at the tube PM (see below).

**Inorganic anions enter the tube via an unidentified mechanism**

An anion influx, most notably carried by chloride, was measured at the tube region in lily and tobacco (Zonia et al., 2002). This influx goes against the expected electrochemical gradient, as estimated using data published in Weisenseel and Wenisch (1980), Zonia et al. (2002) and Messerli et al. (2004). This implies that anions enter the tube via an active or coupled mechanism. No further experimental data is available on anion absorption by the pollen tube, but it is tempting to hypothesize that anions enter the tube region via a H⁺-anion co-transporter, as they do in other plant cells. Several molecular candidates are present in the pollen transcriptome.

**Different domains, different mechanisms: a passive ion transport system in the apical plasma membrane**

From the previous paragraph it stems that most molecular components that carry out the ionic fluxes in the pollen tube system still remain to be characterized. Some predictions, however, can be tested from its expected behavior.

With no H⁺-pump energization, ion fluxes at the tip of the pollen tube are largely expected to be passive. Indeed, Ca²⁺ and H⁺ fluxes flow in the direction of their electrochemical gradients. Anion efflux at the tip has also been shown to be inhibited by direct channel inhibitors (Zonia et al., 2002).

As stated above, H⁺ enters the tip passively. Non-selective cation channels (NSCCs) as well as co-transporters of the H⁺ class may participate to these fluxes. Co-transporters and exchangers could play an important role in H⁺ fluxes at the tip, as suggested by the fact that the family of the CHX transporters is clearly over-represented in pollen, with most of these genes exclusively expressed there (Cellier et al., 2004; Sze et al., 2004; Pina et al., 2005; Bock et al., 2006). CHX transporters are similar to animal H⁺/Na⁺-K⁺ exchangers and have been suggested to play a role in pollen K⁺ homeostasis during growth.

The TRH1 gene codes for a co-transporter K⁺-H⁺ from the AKT/AKUP/HAK family. Its disruption inhibits the root-hair growth (Rigas et al., 2001; Ahn et al., 2004). Increasing the external K⁺ concentration does not revert the phenotype (Desbrosses et al., 2003). This strongly suggests a role of TRH1 in the H⁺ influx across the root-hairs PM which is, like in pollen, restricted to the tip (Gilroy and Jones, 2000; Carol and Dolan, 2002). Members from the TRH1 family are expressed in pollen (Pina et al., 2005).

There is still no molecular evidence for a spatial segregation of K⁺ channels in the pollen tip vs. tube and grain. K⁺ conductance recorded using patch-clamp on the grain or on the tube/tip revealed some electro-physiological differences, suggesting that the channels/transporters active in these regions are different (Fan et al., 1999; Fan et al., 2001; Fan et al., 2003; Griessner and Obermeyer, 2003). Alternatively, one can not discard the hypothesis that the K⁺ transporters and channels may be the same in the tube and the tip regions. On such model, depending on the direction of the potassium flux at the tip, outward channels or transporters, such as SKOR or CHX, could play a dominant role inward transporters, such as SPIK or AKT/AKUP/HAK.

Anions leave the pollen tube by the tip (Zonia et al., 2002). These fluxes are inhibited by pharmacological agents selective for animal chloride channels, and particularly for channels from the CFTR-family (belonging to the ABC-transporter super-family).

No molecular data is available on the identity of the anion channel(s) vs. symporter vs. exchanger at the tip PM. One member from the ABC family, AMRP5, has been reported to play a role in anion permeability regulation in guard-cells (Suh et al., 2007). Although the protein was not shown to mediate anion transport, proteins from the family are interesting candidates to the anion channels of pollen. AMRP5, in addition to two other members of the MRP-family, are expressed in pollen (Pina et al., 2005). Recently, a gene, SLAC1 which is essential for cellular anion homeostasis, has been identified in *Arabidopsis*. SLAC1 is a distant homologue of bacterial and fungal dicarboxylate/ malic acid transporters. Its disruption affects stomatal closure and decreases anion conductance in these cells, suggesting that it encodes for an anion channel (Negi et al., 2008; Vahisalu et al., 2008).

Ca²⁺ enters pollen tubes almost exclusively by the tip (Kuehtreiber and Jaffe, 1990; Pierson et al., 1994; Malhó et al., 1995; Messerli and Robinson, 1997; Messerli et al., 1999).
Channels must mediate these Ca$^{2+}$ influxes. This statement is based on pharmacological as well as electrophysiological results. Hence, as recorded by vibrating probes, Ca$^{2+}$ fluxes are sensitive to a broad spectrum of Ca$^{2+}$ channel inhibitors (nifedipin, Gd$^{3+}$, La$^{3+}$, verapamil, etc.; see e.g. Reiss and Herth, 1985; Geitmann and Cresti, 1998). On the other hand, single-channel traces of channels permeable to Ca$^{2+}$ have been recorded on pollen protoplasts (Dutta and Robinson, 2004; Wang et al., 2004). The channel described by Wang et al. (2004) is inhibited by La$^{3+}$ as well as by Gd$^{3+}$ and activated by hyperpolarization. Dutta et al. (2004) described a channel strongly selective for Ca$^{2+}$ over K$^+$ (pCa$^{2+}$/pK$^+$=98) and activated by membrane stretch. Macroscopic Ca$^{2+}$ currents activated by hyperpolarization have been described in *Lilium davidii* and *Arabidopsis* grains as well as in *Pyrus pyrifolia* tube protoplasts (Shang et al., 2005; Qu et al., 2007; Wu et al., 2007). The selectivity of these currents, as well as their physiological role remains to be elucidated.

The molecular identity of the channels responsible for the Ca$^{2+}$ entry into the pollen in particular, and in plant cells in general, is largely unknown, and identification of Ca$^{2+}$-selective channels, or cation-channels permeable to Ca$^{2+}$ in plants is one of the hot topics in the field (Hetherington and Brownlee, 2004).

Recent studies have pointed out three families of cation channels which share strong sequence homology with animal channels and constitute good candidates to account for Ca$^{2+}$ influxes through the tip PM: the cyclic-nucleotide gated channels (cNGC), the ionotropic amino-acid receptors (iGluR) and the annexines (see Demidchik and Maathuis, 2007). The disruption of a member of the cyclic-nucleotide-gated channels family (cNGC18) induces male sterility, and the expression of this channel in *E. coli* increases Ca$^{2+}$ uptake by the bacteria, suggesting that cNGC18 may participate in calcium influxes at the tip (Frietsch et al., 2007).

**Regulation of ion transport systems in the pollen tube**

The pollen tube polarization correlates with a sub-cellular patterning of central biophysical parameters such as Ca$^{2+}$ concentration, pH, and hypothetical hydrostatic and electric fields. These parameters display gradients along the pollen tube/ tip that are necessary for cellular growth. As described above, ion transporters play a central role in the local control of these parameters and in the establishment of the gradients. On the other hand, they are regulated by these factors. By consequence, ion transport systems should be strongly integrated, and a full understanding of the way they work implies the characterization of these complex regulatory connections. If these imply complete regulatory loops, in which transporters may be simultaneously triggers and effectors of a specific ionic choreography in space and time, the place is reserved for them to perform a crucial and fundamental level of developmental regulation.

**Electrical membrane potential**

The electrical membrane potential ($E_m$) integrates all the ion fluxes at the PM and is thereby a key parameter in cell biology. $E_m$ plays a major role in ion transport regulation and consequently in cell sensing and adaptation (i.e. response) to environment. This was well demonstrated, for example, in the guard-cell system (Thiel et al., 1992; Roelfsema and Hedrich, 2005). In pollen tubes, data on the transporter regulation by $E_m$ is scarce, but the hints are multiple.

K$^+$ conductance (Obermayer and Blatt, 1995; Fan et al., 1999; Fan et al., 2001; Fan et al., 2003; Griessner and Obermeyer, 2003; Becker et al., 2004) and K$^+$ channels (Mouline et al., 2002) are regulated by $E_m$ in pollen.

Ca$^{2+}$ channels have been identified by patch-clamp in the pollen grain. Among them, one has been shown to be activated by hyperpolarization (Wang et al., 2004). At the macroscopic level, hyperpolarization-activated currents have been reported (Shang et al., 2005; Qu et al., 2007; Wu et al., 2007). In root-hairs, the main Ca$^{2+}$ conductance is also activated by hyperpolarization (Very and Davies, 2000). While without positive identification yet, anion channels might also prove to be voltage-gated (Barbier-Brygoo et al., 2000).

**Membrane stretch**

Membrane stretch has been proposed to play a role in the pollen tube growth regulation for a long time (Feijó et al., 1995), mostly based on the striking effect of typical (but not specific) stretch channel inhibitors, like the lanthanides (La$^{3+}$, Gd$^{3+}$). Supporting this idea, stretch-gated channels have been proposed, namely a K$^+$ and a Ca$^{2+}$ channel (Dutta and Robinson, 2004). Proper characterization of stretch-activated channels is still a matter of some debate, and confirmation of these channel activities by other groups would help to settle this concept. Consistent with the concept of a mechanical triggering of some sort is the finding of a Ca$^{2+}$ channel regulated by actin (Wang et al., 2004). Although the two mechanisms (stretch- and actin- regulation) are not related at the molecular level, both are dependent on mechanical forces, and both could be engaged in similar processes in the pollen growth.

**pH**

As any other *bona fide* globular protein, transporters are sensitive to extracellular and cytosolic pH.

H$^+$-ATPases are regulated by both cytosolic pH and H$^+$ electro-chemical gradient across the PM (Palmgren and Christensen, 1994). K$^+$ conductance is activated by an extracellular acidification in the pollen grain and tube (Fan et al., 1999; Fan et al., 2001; Fan et al., 2003; Griessner and Obermeyer, 2003; Becker et al., 2004). At the molecular level, two *Shaker* channels are expressed in the pollen tube; SPIK and SKOR are sensitive to external acidification. Whereas SPIK is activated (Mouline et al., 2002), SKOR is inhibited by external protons (Lacombe et al., 2000). Moreover, the SKOR channel is also inhibited by internal acidification (Lacombe et al., 2000). In contrast, the *AtTPK4* channel, also involved in the pollen tube K$^+$ conductance, is blocked by an internal acidification (Becker et al., 2004).

**Potassium**

The consideration of K$^+$ as a regulator of plant ion transporters is new. Recently, two transporters expressed in pollen have been shown to be regulated by K$^+$: a proton-pump (Buch-Pedersen et al., 2006) and the SKOR K$^+$ channel (Johansson et al., 2006; Liu et al., 2006).

**Ca$^{2+}$ and post-translational modifications**

Many transporters are regulated by Ca$^{2+}$: directly via fixation on the protein core (Becker et al., 2004), or indirectly, via the complex signaling pathways downstream of Ca$^{2+}$ elevations. For example, the AKT1 mRNA has been detected in pollen by RT-PCR, and this channel is known to be regulated by Ca$^{2+}$-dependent phosphorylation (Li et al., 2006).

The regulation of pollen transporters by Ca$^{2+}$ is poorly docu-
mented, but given its central influence in many physiological responses, it is generally accepted that it must play some sort of direct or indirect role. Recently, the AtTPK4 K⁺ channel has been shown to be regulated by Ca²⁺ (Becker et al., 2004).

**Ion transporter network and cross-signaling: the need for models and systems approaches**

The regulation parameters we just discussed, though intrinsically important, are unlikely to fill the complete list of possible effectors and affecters of ion channels. Of sure importance, but even less characterized, should be the influence of the endo/exocytosis and membrane flow systems in general in the correct targeting and assignment of transporters. And the only sure thing is that most factors will interact at some level to create the specific dynamical equilibrium that we recognize as a stable phenotype.

The Ca²⁺ channel network offers a good example of the complexity of this multi-signaling interaction. Ca²⁺ channels are sensitive to stretch, membrane potential, and probably cytosolic pH and Ca²⁺. In

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**Fig. 4. The proton-pump plays a central role in proton homeostasis and proton gradient establishment in pollen tube.** A H⁺-pump system is present and active at the tube plasma membrane (PM) while neither its presence nor its activity seem to be detected at the tip PM. The spatial patterning of the H⁺-pump activity may be a key point in the pollen tube polarization establishment by defining two transport domains at the pollen PM: an active transport system at the tube, and a passive one at the tip. In turn this may allow the establishment of cytosolic gradients of H⁺ concentration, which may work as a second messenger. (A) Imaging of H⁺ concentration inside one lily growing pollen tube. The picture was taken in wide field using the BCECF-dextran ratiometric dye (Feijó et al., 1999). Note the existence of an alkaline domain in the sub-apical region. The projective nature of the imaging method used does not allow the resolution of the precise geometry of this domain, which may exist at the core, or as a sub-membranar torus. (B) H⁺ fluxes around a lily pollen tube recorded using a H⁺-specific vibrating probe. The maximum intensity of H⁺ efflux at the tube spatially correlates with the alkaline domain shown in (A). Note however that the fluxes in this species are of much less intensity than in tobacco, while in contrast, the tube is rather thicker (Feijó et al., 1999). (C) Model of proton homeostasis in lily. This model was created to account for the data exposed in (A,B), and is based on the assumption that the H⁺-pump should be absent from the tip (Feijó et al., 1999). Because H⁺ ions enter at the tip and leave at the tube, this would create a pH gradient inside the cytoplasm (i.e. acidic tip). The activity of the H⁺-pump on the tube PM may locally alkalize the cytoplasm and create thereby the alkaline domain visible in (A) and the differential sub-membranar domains depicted in Fig. 3A. The existence of similar H⁺ loops in root hairs has led to the prediction that this could constitute a more generalized mechanism of polarity in apical growing cells (Palmgren, 2001; Feijó et al. 2004). (D) Cloning of the H⁺-pump Nt AHA (Nicotiana H⁺-ATPase) from tobacco, confirmed the essential mechanistic prediction of this model. Fluorescence imaging of a growing pollen tube expressing a Nt AHA-GFP chimera (Certal et al., 2008) shows that Nt AHA is selectively excluded from the PM of the apex. (E) Cytochemical analysis of ATPase activity in massulate pollen of the orchid Ophrys lutea (Feijó, 1995). Cerium precipitates corresponding to the activity of K⁺ dependent ATPases are specifically located in plasma membrane of the pollen grain (GP) but absent from the tip (TP). (F) H⁺ fluxes around a tobacco pollen tube as measured with a H⁺-specific vibrating probe. The pattern is coincident with the one of distribution of Nt AHA shown in (D), i.e., where the pump is present there is a net efflux, where the pump is absent, H⁺ leak in the tube. As in lily, H⁺ enter the tip and leave the tube and the grain. H⁺ efflux patterns in lily and in tobacco are different along the tube PM both qualitatively, but mostly in quantitative terms (compare (B,F)). These differences are in good accordance with the differences detected in the imaging of intracellular pH in tobacco as compared with lily (Michard et al., 2008).
Ca²⁺ and H⁺ oscillations play a key role in the guard-cell signaling network, via comparable relative amplitudes, they seem to be coordinated by that all these oscillations share the same central period and (Holdaway-Clarke and Hepler, 2003). It may be involved in the generation – or modulation – of oscillations which suggests that H⁺ transporters, or H⁺-regulated-transporters External pH modulates the oscillation parameters of the cells, the tip are directly involved in the generation of oscillations.

A role for ion transporters in the generation of pollen tube oscillations?

Depending on the growing condition, the pollen tubes of the majority of species so far known will display some sort of periodicity, usually as sustained growth oscillations (Feijó et al., 2001). Almost all physiological parameters of the cell oscillate: the vesicles/ cytoskeleton dynamics (Parton et al., 2001; Hwang et al., 2005; Lovy-Wheeler et al., 2006), ion cytoplasm concentrations (Ca²⁺ and H⁺ gradient; Holdaway-Clarke et al., 1997; Feijó et al., 1999), ion fluxes, and on the top of all, growth. To the extent that all these oscillations share the same central period and comparable relative amplitudes, they seem to be coordinated by the same oscillator (Feijó, 1999; Moreno et al., 2007). For example, ion fluxes at the tip of the pollen tube are strongly correlated, but not in phase, with growth oscillations, fluxes and intracellular concentrations oscillations of Ca²⁺ (Pierson et al., 1996; Holdaway-Clarke et al., 1997; Messerli et al., 1999; Messerli et al., 2000), H⁺ (Feijó et al., 1999; Messerli et al., 1999; Lovy-Wheeler et al., 2006) and Cl⁻ (Zonia et al., 2002)(see Figs. 2, 3).

The mechanism responsible for pollen growth oscillation (the “pacemaker”) is unknown. A role for cell-wall (Messerli and Robinson, 2003; Dutta and Robinson, 2004) or reduction potential (Cárdenas et al., 2006) have been proposed. Passive transport systems have been shown – or proposed – to be able to generate spontaneous oscillations (Gradmann et al., 1993). These oscillations play a key role in the guard-cell signaling network, via Ca²⁺ and H⁺ (Allen et al., 2000; Allen et al., 2001). The transport system at the tip of the pollen is expected to be passive, and a similar mechanism of oscillation generations could occur. Ca²⁺ channels inhibitors affect the oscillation properties of pollen. (Geitmann and Cresti, 1998), suggesting that Ca²⁺ channels at the tip are directly involved in the generation of oscillations. External pH modulates the oscillation parameters of the cells, which suggests that H⁺ transporters, or H⁺-regulated-transporters may be involved in the generation – or modulation – of oscillations (Holdaway-Clarke and Hepler, 2003).

Conclusions

A model for pollen electric polarization establishment

Throughout this review, we have highlighted the importance of ion transporters in the establishment of gradients along the growing pollen tubes. These gradients are essential for the regulation of growth. On the other hand, the polarization of ion fluxes has therefore an obvious physiological role on the spatial patterning of the growing cell, and the establishment and maintenance of its polarity.

The data we reviewed makes it tempting to put up a model for the establishment of electrical polarity in growing pollen tubes (Fig. 1 B,C). In a non-polarized cell, and when the PM is at the resting potential, the ion fluxes that cross it altogether induce no net charge flux. The PM can not support any strong charge gradient (i.e. strong electrical polarization), and once the steady-state Ems reached, the charge efflux driven by the proton-pump is exactly compensated by other ion fluxes. Cation influx (namely K⁺) and anion efflux via transporters take part in this charge compensation. The PM is permeable to H⁺, and a “proton shunt” (i.e. inward H⁺ flux through the lipid bi-lager, or specific H⁺ channels) exactly compensates the H⁺-pump charge movement in addition to cation influxes/ anion effluxes.

The pollen tube is usually hyperpolarized, and notably above the K⁺ equilibrium potential (Weisenseel and Wenisch, 1980; Mouline et al., 2002). This hyperpolarization can be linked to the H⁺ efflux recorded in this region (Feijó et al., 1999; Cerf et al., 2008), and is attributable to H⁺-pump activity (Weisenseel and Wenisch, 1980; Cerf et al., 2008). Consequently there should be a charge depletion in the close vicinity of the H⁺-pump. The compensation of this charge depletion can be achieved by two competitive mechanisms: (i) the “proton shunt” and (ii) the influx of charges from external media across the tip PM and cytoplasm that electrically links tip and tube. The relative role of these two mechanisms in H⁺-pump electrical compensation depends respectively (i) on the conductance of the tube PM to H⁺ (GΩ⁻¹ range) (ii) and on the electrical conductance of the tip ion-transport system (MΩ⁻¹ range) plus the cytoplasm (kΩ⁻¹ range). The relative high electric conductance of both the cytoplasm and the tip PM un-favors the “proton shunt” and induces a net charge flux inside the pollen system. While probably representing a minimal model, we highlight that this model does fit into context all the available data, while allowing a number of testable hypotheses. It is tempting to think that if this model is correct, it would imply the existence of an intracellular physical field through the steady flux of H⁺ and other charged particles. It may be argued that such mechanism should have a theoretical sensitivity to minute intra and extracellular perturbations of the field. If properly connected to the canonical signaling networks, it could constitute a novel fundamental scheme for cell polarization and spatial organization.

A role of transport system polarization in nutrient uptake efficiency?

In addition, the unique transport system of the polarized cell may have other physiological roles. The co-existence in the same cell of an active transport system (tube) and a passive one (tip) may be responsible for the remarkable efficiency of the pollen tube, but possibly also for root hairs, in the nutrient uptake, particularly K⁺.

K⁺ is one of the major limiting factors of plant cell growth (Epstein, 1976). The persistence of a sustained growth of pollen under low K⁺ concentrations is striking, and suggests that pollen has an extremely efficient K⁺ uptake system (Mouline et al., 2002). Channels, driven by the H⁺-pump, play a significant role in the high-affinity K⁺ uptake in pollen. This K⁺ absorption system
depends on the H+-pump efficiency to hyperpolarize the PM. H+-pump activity, on the other hand, is regulated indirectly by post-translational modifications (Pettit et al., 2001), and directly, by cytoplasmic factors such as pH (Palmgren and Christensen, 1994; Luo et al., 1999; Palmgren, 2001; Sondergaard et al., 2004). In pollen, AHA3 has notably been shown to be regulated by internal pH (Palmgren and Christensen, 1994; Robertson et al., 2004). H+-influxes at the tip affect pH in the tube, and acidify the cytosol near the H+-pump. This should activate the H+-pump, by changing the conformation of the protein, and by decreasing the H+-electrochemical gradient across the plasma membrane. Thereby, the passive transport system at the tip is expected to activate the active transport system at the tube and favor nutrient absorption. We note that this mechanism should have direct implications in the unique capacity these cells have to grow at such fast growth rates.

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Role of ion fluxes on pollen tube growth


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