

Signaling with Ions: The Keystone for Apical Cell Growth and Morphogenesis in Pollen Tubes^{1[OPEN]}

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Pollen tubes (PTs) are one of the best characterized plant cell types in many respects. The identification of key players involved in tube growth offers the perspective of an integrative understanding of cell morphogenesis processes. One outstanding feature of PTs is their prominent dependence on ion dynamics to promote and regulate growth. Many reports have identified and characterized membrane transport proteins, such as channels, transporters, and pumps, as well as their regulatory mechanisms, some of which themselves are dependent on ions such as Ca^{2+} and H^+ . The signaling network that governs growth is based on a strict spatial distribution of signaling molecules, including apical gradients of Ca^{2+} , H^+ , and reactive oxygen species. A central role for ion homeostasis, and more generally membrane transport systems, is proposed to underlie the spatiotemporal establishment of the signaling network that controls the PT self-organization and morphogenesis. Here, we review the latest progress on understanding tube growth from the perspective of membrane transporters and ion homeostasis. The ongoing molecular characterization of the Ca^{2+} -signaling pathways, as well as the recent identification of female external cues and corresponding receptors on the pollen that control growth orientation, offer a firm biological context to boost the field even further.

POLLEN TUBES AS A TAILORED MODEL FOR STUDYING ION DYNAMICS AT THE CELL BIOLOGY LEVEL

Pollen tubes (PTs) have long been considered outstanding models for cell biology for a variety of reasons. On the one hand, they display dramatic features at the level of cell polarity, cytoskeleton dynamics, growth rates, membrane recycling, cell-cell interaction mechanisms, etc. (Cheung and Wu, 2007; Michard et al., 2009; Qin and Yang, 2011; Hepler, 2016). On the other hand, their study is backed up by extensive databases on

transcriptomics and proteomics on practically all of its biological contexts (Honys and Twell, 2003; Pina et al., 2005; Borges et al., 2008; Qin et al., 2009; Boavida et al., 2011; Mayank et al., 2012; Pertl-Obermeyer et al., 2014; Lang et al., 2015). All these features define a unique cell type so evolutionarily streamlined to fast growth and sperm delivery (Williams, 2008) that it remained basically conserved as the only gametophyte developmental end product for male function since the

ADVANCES

- Pollen tube growth is strictly dependent on ion dynamics, and this property has been used to detect phenotypes of novel ion transport proteins.
- Ion fluxes and cytosolic gradients of concentration have been mechanistically associated with the action of specific transporters, especially for protons.
- GLR channels and CNGCs have been established as cation-transporting channels with a role in Ca^{2+} homeostasis and signaling in growing pollen tubes.
- Ca^{2+} and ROS signaling networks are interconnected and are key elements to regulate RHO GTPases, NADPH oxidases, and Ca^{2+} downstream signaling cascades.
- Specific temporal patterns of cytosolic Ca^{2+} have been implicated in various steps of fertilization, suggesting the existence of cell-cell communication events that are mediated by Ca^{2+} signaling.

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Cretaceous (Rudall and Bateman, 2007). PTs in angiosperms can grow up to $4 \mu\text{m} \cdot \text{s}^{-1}$ and are characterized by the periodic formation of callose plugs that isolate older parts of the tube to die so that growth can be maintained continuously restricted to the apical end (for review, see Boavida et al., 2005a, 2005b). In plants, PTs share the same type of apical growth mechanism with root hairs, a fact that is reflected at the molecular level by the existence of an apical signature in transcriptomics profiles (Becker et al., 2014). Root hairs, however, grow slower than PTs. In addition, root hair length is controlled through a signaling network involving ROOTHAIR DEFECTIVE SIX-LIKE (RSL) transcription factors (Honkanen and Dolan, 2016). In spite of those main differences, the apical growth of root hairs and PTs is characterized by apical exocytosis of new cell wall material, similar ion gradients, fluxes at the tip, and a mechanism depending on actin cytoskeleton supporting cell elongation (Gu and Nielsen, 2013; Ketelaar, 2013; Mendrinna and Persson, 2015; Mangano et al., 2016).

With so many peculiarities and extreme adaptations to function, the applicability of PT-specific mechanisms to other plant somatic cells, namely diffuse growing ones, is not always straightforward. This assumption is clearly reflected in the well-differentiated transcriptomic profiles between PTs and those of other organs and tissues (for snail-view representations, see Becker et al., 2003; Pina et al., 2005). The one feature in PTs that stands out the most is their strict dependence on ion dynamics to grow and sustain their functions (Michard et al., 2009). Different ions, namely calcium (Ca^{2+}), protons (H^+ or pH), and chloride (Cl^-), form stable/standing gradients of cytosolic concentration in the clear zone (Fig. 1). Of relevance, these spatial patterns and their temporal and spatial variations or choreographies, correlate remarkably well with the intracellular structure of PTs, be it the grading of organelle sizes defining the so-called clear zone, the cytoplasmic reverse fountain (Cheung and Wu, 2007; Hepler and Winship, 2015), and, to some extent, the definition of the membrane-recycling domains in the tip (Parton et al., 2001; Bove et al., 2008; Kost, 2008; Bloch et al., 2016; Wang et al., 2016; Fig. 1). The existence of these ion gradients as a putative central coordinating mechanism for cellular growth and morphogenesis in PTs has been conceptualized elsewhere (Feijó et al., 1995, 2001; Michard et al., 2009; Daminelli et al., 2017). Largely believed to be generated from plasma membrane (PM) activity, the ion choreography of PTs is easily traceable by noninvasive methods such as ion-specific vibrating probe electrophysiology and ion-specific probe imaging to show nearly perfect correlation with growth variations while also allowing one to score for very subtle phenotypes hardly detectable in other nongrowing cells (Michard et al., 2011).

Of relevance, growth rate, ion fluxes, and concentrations may oscillate in PTs, as well as during root hair growth. Some studies present the choreography of ion fluxes and intracellular ion concentrations by a relative

lag time during a growth period in PT (for review, see Holdaway-Clarke and Hepler, 2003; Hepler et al., 2013) and root hair (Monshausen et al., 2007, 2008). In such studies, the minimum pH or maximum Ca^{2+} oscillations and growth peak display a time lag of a few seconds in both PT and root hairs, suggestive of similar regulation mechanisms of growth. Of note, the flux of Cl^- was found to be in phase with growth (Zonia et al., 2002). Nevertheless, different estimates of advances and delays have been collected in a variety of biological systems like lily (*Lilium longiflorum*), tobacco (*Nicotiana tabacum*), petunia (*Petunia hybrida*), less in Arabidopsis (*Arabidopsis thaliana*), using imaging techniques (differential interference contrast, wide-field or confocal fluorescence), and electrophysiology methods in such ways that comparisons of the published delays and proposed sequences of events are subject to potential distortions (Portes et al., 2015; Daminelli et al., 2017). Last but not least, correlation does not imply causation, and not much can be deduced from those studies, particularly because we do not know the kinetic properties of key reactions within the networks, such as molecular diffusion, protein phosphorylation, exocytosis, etc. (Daminelli et al., 2017)

Figure 1 highlights the peculiar correlation between ion dynamics and cell structure. Spatial correlations between features of the cytosolic gradients (Fig. 1, B, C, and E) and other cellular structures are conspicuous and easily observed at the level of zonation of organelles along the clear zone (Fig. 1A) or the actin cytoskeleton (Fig. 1D). Characterizing the transport molecules that generate these gradients may be a first step in their manipulation and eventually may test the hypothesis that spatial correlations are not a mere phenomenological coincidence but may actually be causal and part of a network of regulatory feedback loops. One first step in that direction is the establishment of a functional correlation between the transport molecules and the predicted outcome of their activity in terms of ion dynamics, whether at the level of cytosolic concentration or of membrane transport. One such example is also offered in Figure 1, where the localization of the H^+ -ATPase NICOTIANA TABACUM AUTO-INHIBITED H^+ -ATPASE (NtAHA1) (Fig. 1F) correlates perfectly with the existence of intracellular pH domains (Fig. 1E) and extracellular H^+ fluxes (Fig. 1G; Certal et al., 2008; Michard et al., 2008). The fact that this crucial pump is segregated from the tip PM triggers a number of testable models and by itself already defines an experimental paradigm offered uniquely by PTs.

In Arabidopsis, more than 800 transporter transcripts have been identified in pollen using the ATH1 mRNA microarray (Pina et al., 2005; Bock et al., 2006), and this overrepresentation is confirmed by RNA sequencing in Arabidopsis and lily (Loraine et al., 2013; Lang et al., 2015). This is perhaps one of the reasons why PTs have been widely explored in recent years for phenotyping an increasing repertoire of channels, transporters, and pumps, rendering the vegetative cell of the PT likely one of the best studied cells in plants in terms of ion

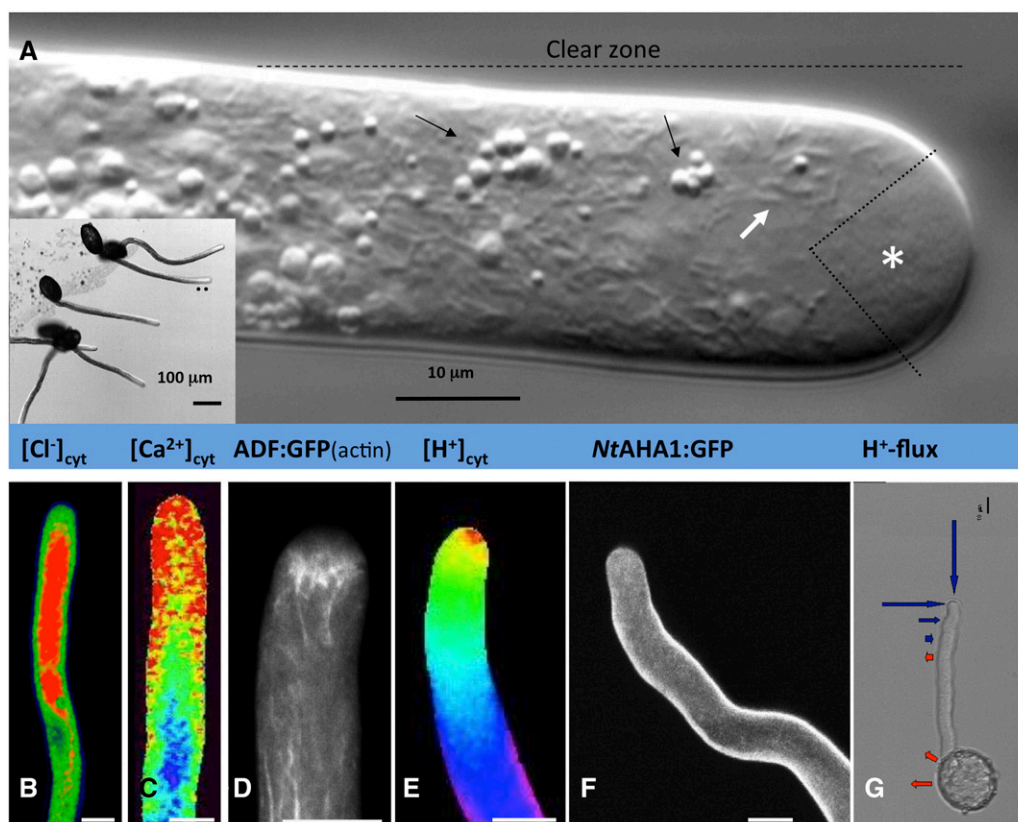


Figure 1. The ion dynamics regulatory paradigm of the PT structure is supported by strong spatial correlations. A, Lily PTs (differential interference contrast imaging) display the quintessence of the dramatic polarization of these cells. Actin-driven reverse-fountain cytoplasmic movement drives organelles to the tip by the periphery and back through the tube core. Yet, this back-turning movement occurs some 20 to 50 μm back from the tip, resulting in a pattern best described as if there is a size-sorting or sieving mechanism, first dispatching back big starch plastids (black arrows), then mitochondria (white arrow) and other larger endomembrane formations, and only allowing small exocytic vesicles to the very tip (asterisk). This pattern generates two clearly observable cytologic domains: the clear zone, defined by the absence of large plastids and many times seen at low magnification (double asterisk in the inset), and an inverted cone (Lancelle et al., 1987) of small vesicles that, contrary to the rest of the tube shank, move in a nonorganized Brownian-like motion (dashed lines on the tip). B to G, In tobacco PTs, these domains are remarkably correlated with cytosolic ion concentrations of Cl^- (B), Ca^{2+} (C), and H^+ (E) when live imaged with specific fluorescent genetic probes. The acidic tip (E) very closely defines the inverted cone and is also correlated with a lower concentration of Cl^- . In tobacco, the inverted cone also seems to be lined up by a concentration of small, highly dynamic actin filaments (D) that are often organized as a basket or fringe (Rounds et al., 2014). The clear zone is correlated with both the fading of the Ca^{2+} and the acidic gradients focused on the tip. The base of the clear zone also defines the appearance of the long actin filaments that support organelle streaming. A causal correlation between a specific transporter distribution and cytosolic concentration of an ion is best seen for H^+ , where the localization of the most highly expressed P-type ATPase in tobacco, NtAHA1 (F; Certal et al., 2008), closely correlates with the extracellular H^+ fluxes (G; effluxes in red and influxes in blue) and cytosolic pH (E). H^+ effluxes, on the other hand, correlate with the presence of the chimeric NtAHA1::GFP protein, and influxes correlate with their absence on the tip. Last but not least, the acid tip (red in E) correlates with the largest influxes and the submembranar alkaline linings (purple in E) correlate with effluxes. Bars = 10 μm . (The image in A is by N. Moreno, adapted from Taiz et al. [2015] and Smith et al. [2009]; the image in B is adapted from Gutermuth et al. [2013]; the images in C and E are adapted from Michard et al. [2008]).

dynamics. Figure 2 and Table I summarize this accumulated knowledge. They incorporate not only genes that have already been characterized but also genes that can be predicted from transcriptomics, proteomics, and comparative physiology to play roles in PT growth. In this review, we focus on organizing or systematizing this growing repertoire, focusing on three different angles. (1) Is this knowledge sufficient to define regulatory mechanisms around a specific ion? (2) What are the

downstream targets of specific ions? And (3) how do cellular processes feed back to regulate ion transport?

OPPOSING FORCES: TURGOR AND CELL WALL DEPOSITION

When growing PTs or root hairs stop in response to an osmotic shock, the exocytosis of vesicles ensuring cell wall deposition continues at the tip (Schroeter and

Table 1. Summary of molecular data available on the physiological role of ion or turgor-related membrane transport proteins in PT growth, gathered by function (pumps, channels, cotransporters, and aquaporins) and selectivity (sugars, anions, potassium, and cations)
 For each transporter and corresponding genomic identifier, the molecular function and the biological system in which it has been established are indicated as well as the intracellular localization and the localization method(s) used. Physiological relevance is defined by phenotypes of knockdown or overexpressing lines, when available, or other physiological traits. Putative genes are indicated when (1) transcriptomics indicated pollen selective and high expression and (2) function has been defined for the gene family in other tissues. n/a, Not available.

Name	Locus Identifier	Function (System ^a)	Localization ^b (Method ^c)	Physiological Relevance ^d	Reference
Pumps					
NtAHA1	AY383599	P-type ATPase; proton pump (P)	PT, PM, shank (FP)	OX: slowed growth rate, callose plug deformation	Certal et al. (2008)
NpPMA5	AY772462 AY772468	P-type ATPase; proton pump (Y)	Shank PM (I)	?	Lefebvre et al. (2005)
LtHA1	AY029190	P-type ATPase; proton pump (P)	PG, PM (PC)	H ⁺ currents under patch clamp	Gehwolf et al. (2002)
AtAHA3	At5g57350	P3A-type ATPase; proton pump (P)	PM?	Expressed during pollen development (GUS); KO: lethal	Robertson et al. (2004)
AtAHA6	At2g07560	P3A-type ATPase; proton pump?	PM?	Putative, based on expression levels (AHA8), homology with Nt and Lt, and pollen selective	Pina et al. (2005); Bock et al. (2006); Lang et al. (2015)
AtAHA7	At3g60330			(AHA6, AHA7, and AHA9)	
AtAHA8	At3g42640			KO: partial male sterility	Schiott et al. (2004)
AtAHA9	At1g80660				
AtACA9	At3g21180	P2B-type ATPase; calcium pump (Y)	PM (FP)		
AtVHA-E1	At4g11150 At3g08560	Vacuolar H ⁺ -ATPase (P)	E1, vacuoles and endosomes of sperm cell; E2, vegetative cell, pollen specific; E3, vegetative cell and sperm cell vacuole (FP)	E1, KO is embryo lethal; E2, KO has no phenotype; E3, partially redundant to E1, possible stress response implications	Strompen et al. (2005); Dettmer et al. (2010)
AtVHA-E2	At1g64200				
AtVHA-E3					
Suc transporters					
AtSUC1	At1g71880	Suc carrier (P)	PM, around callose plugs and cytoplasm near PT tip (FP)	KO: reduced pollen germination rate	Stadler et al. (1999); Sivitz et al. (2008)
OsSUT1	Os03g07480	Suc transporter (P)	?	KO: impaired germination rate	Hirose et al. (2010)
LeSUT2	Solyc11g017010	Suc transporter (P)	PT, PM (I)	AS: decreased amounts of soluble sugars, inhibited PT growth	Hackel et al. (2006)
CsHT1	GenBank HQ202746	Hexose transporter, high affinity for Glc (Y)	PM (FP)	OX: higher pollen germination rate, increased tube growth; AS: inhibited germination and elongation, fewer seeds	Cheng et al. (2015)
AtSWEET8/ AtRPG1	At5g40260	Suc transporter (P, H, Y)	PM (FP)	KO: male fertility phenotype, microsporogenesis, exine formation and cell integrity	Guan et al. (2008); Chen et al. (2010); Sun et al. (2013)
Anion channels and transporters					
AtSLAH3	At5g24030	Anion channel (P, O)	PM (FP)	Voltage clamp: regulated by AtCPK2 and AtCPK20	Gutermuth et al. (2013)
AtMSL8	At2g17010	Anion channel (O)	PM, endomembranes (FP)	WT: mechanosensitive; KO: improved germination; OX: inhibited germination (negatively regulates)	Hamilton et al. (2015)

(Table continues on following page.)

Table 1. (Continued from previous page.)

Name	Locus Identifier	Function (System ^a)	Localization ^b (Method ^c)	Physiological Relevance ^d	Reference
AtCCC1	At1g30450	Cl ⁻ /cation cotransporter (O)	PM, Golgi (FP)	Pollen expression; KO: aborted siliques, seed-set reduction	Colmenero-Flores et al. (2007); Kong et al. (2011); Henderson et al. (2015)
Potassium channels					
AtSPIK (AtAKT6)	At2g25600	K ⁺ channel (C)	PM (PC)	WT: voltage dependent, inward currents, CsCl inhibition; KO: disrupted pollen germination, slower tube growth, fertility affected	Mouline et al. (2002)
LiIKT1	Gene n/a; protein A3RG92	K ⁺ channel (P, Y)	Cytoplasm, punctate; PM in tobacco epidermis (FP)	?	Safarian et al. (2015)
AtTPK4	At1g02510	K ⁺ channel (P, O, Y)	PM (PC)	WT: regulated in pH, calcium-dependent manner	Becker et al. (2004)
AtSKOR	At3g02850	Outward-rectifying K ⁺ channel	?	Putative, pollen selective	Pina et al. (2005)
Cation channels and transporters					
AtCNGC7	At1g15990	Cation channel?	AtCNGC7: tip PM during tube emergence, PT shank during elongation (FP)	WT: pollen fertility, initiation of PT growth; double KO: male sterile	Tunc-Ozdemir et al. (2013a)
AtCNGC8	At1g19780	Cation channel?	?	WT: PT germination and growth during stress; KO: reduced competitive fitness, fewer seeds, low pollen transmission	Tunc-Ozdemir et al. (2013b)
AtCNGC16	At3g48010	Cation channel?	?	WT: Ca ²⁺ influx; KO: sterile	Frietsch et al. (2007); Gao et al. (2016)
AtCNGC18	At5g14870	Ca ²⁺ permeation (E), nonselective cation channel (H)	PT apical PM (FP)	Putative, pollen selective	Pina et al. (2005); Morris et al. (2008)
AtCAX4	At5g01490	Cation/Ca ²⁺ exchanger	Endomembranes	WT: Ca ²⁺ influx, PT growth, and morphogenesis; KO: AS, partial male sterility	Michard et al. (2011)
AtCAX9	At3g14070	Cation channel, Ca ²⁺ permeable (P)	Ca ²⁺ influx at PT tip PM (VP)	KO: decreased growth rate, partial male sterility	Michard et al. (2011)
AtGLR1.2	At5g48400	Cation channel, Ca ²⁺ permeable (P)	?	KO: disrupted the formation of mitochondria and Ca ²⁺ uptake	Teardo et al. (2015)
AtGLR3.7	At2g32400	Cation channel?	?	?	Hou et al. (2014); Yuan et al. (2014)
AtGLR3.5	At2g32390	Cation channel?	Mitochondria, chloroplast (FP)	KO: higher free Ca ²⁺ in mitochondria, faster and higher Ca ²⁺ accumulation in response to auxin and extracellular ATP	Stael et al. (2012); Wagner et al. (2015)
AtOSCA1.7	At4g02900	Ca ²⁺ -permeable? osmolarity gated?	PM?	?	
AtMICU	At4g32060	Ca ²⁺ -binding protein (P)	Mitochondria (l, FP)		
H⁺/cation cotransporters					

(Table continues on following page.)

Table 1. (Continued from previous page.)

Name	Locus Identifier	Function (System ^a)	Localization ^b (Method ^c)	Physiological Relevance ^d	Reference
AtCHX19	At3g17630	Cation channel?	PM (FP)	KO: <i>chx17/chx18/chx19</i> mutant pollen normal, reciprocal cross experiments indicate a largely male defect	Padmanaban et al. (2016)
AtCHX13	At2g30240	K ⁺ acquisition, high-affinity K ⁺ uptake (Y)	PM (FP)	Roots and seedlings: KO: sensitive to K ⁺ deficiency; OX: reduced sensitivity to K ⁺ deficiency; no pollen phenotype	Size et al. (2004); Zhao et al. (2008)
AtCHX14	At1g06970	Low-affinity K ⁺ efflux (P, Y)	PM (FP)	KO and double KO <i>chx13/14</i> : root growth sensitive to high K ⁺ ; OX: root growth increase in high K ⁺ ; no pollen phenotype	Size et al. (2004); Zhao et al. (2015)
AtCHX21 AtCHX23	At2g31910 At1g05580	AtCHX23: K ⁺ uptake in a pH-dependent way (E)	Endomembranes (FP)	KO: PT navigation	Size et al. (2004); Evans et al. (2011); Lu et al. (2011)
AtNHX1 AtNHX2	At5g27150 At3g05030	Na ⁺ /H ⁺ antiporter (P)	Vacuole (FP)	Double KO: pollen unaffected; regulates pH and K ⁺ homeostasis	Bassil et al. (2011)
Aquaporins AtNIP4;1 AtNIP4;2	At5g37810 At5g37820	Water and nonionic solute channels (O)	AtNIP4;1, PM and intracellular vesicles in PT, pollen grains; AtNIP4;2, PM and intracellular vesicles of PT only (FP)	KO: fewer seeds, reduced pollen germination and PT length	Di Giorgio et al. (2016)
NIP1;1 NIP2;1 AtTIP1;3	AF440271 AF440272 At4g01470	Water channels (O) Regulation of water fluxes?, water and solute transport (O) Regulation of water fluxes?, water and solute transport (O)	PM? TP of vegetative cell (FP)	Pollen dehydration and dehiscence	Bots et al. (2005)
AtTIP5;1	At3g47440	Regulation of water fluxes?, water and solute transport (O)	TP of sperm cells (FP)	Double KO with AtTIP5;1 revealed poor seed development and silique growth Double KO with AtTIP1;3 revealed poor seed development and silique growth	Soto et al. (2008); Wudick et al. (2014) Soto et al. (2008); Wudick et al. (2014)

^aC, COS cells; E, *Escherichia coli*; H, HEK cells; O, *Xenopus laevis* oocytes; P, plant; Y, yeast. ^bTP, Tonoplast. ^cFP, Fluorescent protein fusion; I, immunodetection; PC, patch clamp; VP, calcium-selective vibrating probe. ^dAS, Antisense line; KO, knockout line; OX, overexpression line; WT, wild-type line.

(NIP) aquaporins were localized in the pollen PM (Lang et al., 2015). Heterologous overexpression of PLASMA MEMBRANE INTRINSIC PROTEIN (AtPIP) aquaporins yielded an increase of the water permeability of lily pollen but no evident functional phenotype (Sommer et al., 2008). Aquaporins from the SIP, TIP, and NIP families were shown to transport water and/or solutes and appear to be involved in PT growth (Ishikawa et al., 2005; Soto et al., 2008; Di Giorgio et al., 2016) and fertilization (Wudick et al., 2014). Interestingly, although not expressed in pollen, it was reported recently that AtPIP2;1 also shows a nonselective cation channel activity (Byrt et al., 2016), a feature that might be found for other members of the aquaporin family.

Ion-driven osmotic changes induce electric potential shifts at the PM in addition to external pH along the PT, and osmoregulation depends on an active transport system driven by the proton pump, through 14-3-3 protein regulation (Pertl et al., 2010). Several H⁺-ATPase pumps are expressed in pollen (Pina et al., 2005; Bock et al., 2006), with AtAHA8 being the most highly expressed and AtAHA6, AtAHA7, and AtAHA9 being pollen specific (Table I). In tobacco, a close homolog of AtAHA6 and AtAHA9, NtNHA1, was found to be localized on the PM but segregated from the tip and involved in tube growth and callose plug formation (Cortal et al., 2008). These pumps are likely to energize the transport of other molecules that underlie turgor in PTs, such as sucrose (Stadler et al., 1999; Goetz et al., 2001). While not necessarily affecting only PT growth, the Arabidopsis AtSUC1 and rice (*Oryza sativa*) OsSUT1 sucrose transporters have defective male gametophyte phenotypes (Sivitz et al., 2008; Hirose et al., 2010). In cucumber (*Cucumis sativus*), the hexose transporter CsHT1 is necessary for PT growth (Cheng et al., 2015). Despite probably being related to microsporogenesis and exine pattern formation, mutants of the PM-localized sucrose transporter AtRPG1/AtSWEET8 display fertility defects (Guan et al., 2008; Chen et al., 2010; Sun et al., 2013).

However, and importantly, ion fluxes such as anions (Zonia et al., 2002) or K⁺ may participate in turgor generation. K⁺ inward conductivities have been recorded by patch clamp and voltage clamp in lily and Arabidopsis (Mouline et al., 2002; Griessner and Obermeyer, 2003; Becker et al., 2004). The inward rectifier AtSPIK channel is involved in PT growth (Mouline et al., 2002), and AtTPK4 mediates nonrectifying currents and also may participate in osmotic regulation of the PT (Becker et al., 2004). For anions, major solutes associated with water movement and turgor in animals and plants, only AtSLAH3 has been characterized in PTs (Gutermuth et al., 2013), but it only accounts for a small percentage of the total anion flux. Yet the demonstration of a role for anion channels in stomatal turgor regulation offers an analogy that could eventually serve as a conceptual template to screen for their identity in PTs (see Text Box 1; Fig. 3). High turgor pressure typically induces the bursting in both hyphae, another tip-growing cell (Money and Hill, 1997), and PTs (Benkert et al., 1997; Amien et al.,

2010). The rupture point always being the tip suggests anisotropy in the cell wall mechanical properties, characterized by a stronger shank. In hyphae, the turgor pressure plays a minor role in polarization; rather, the apical localization of lytic enzymes that loosen the cell wall determines the growth rate and polarity (Money and Hill, 1997). Similarly, PT growth is sustained by the deposition of primary cell wall material at the apex; once deposited at the tip, the wall is subject to a maturation process that stiffens it, creating a gradient of viscosity/elasticity between the growing tip and the nongrowing tube (Hepler et al., 2013; Cosgrove, 2016). Despite discrepancies over the quantification of the mechanical properties of the cell wall (Fayant et al., 2010; Vogler et al., 2012), many biochemical data demonstrate its anisotropic composition and suggest a viscosity gradient along the tube (Steer and Steer, 1989; Geitmann, 2010; Chebli et al., 2012; Hepler et al., 2013). The primary cell wall of the PT deposited at the apex is essentially composed of pectin plus 2% to 3% cellulose (Aouar et al., 2010; Derksen et al., 2011). Pectins are exported as methyl esters and, in parallel, some pectin methyltransferase enzymes (PME) are secreted by the PT and catalyze pectin deesterification. This pectin chemomechanical structure largely determines the growth of the tube, as revealed by pectinase treatment that affects growth properties and induces tube swelling (Parre and Geitmann, 2005) and by the PME mutant *vanguard1* PTs, which are slower and burst precociously (Jiang et al., 2005). Both Ca₂₁ and H₁ either cross-link pectin polymers to induce the formation of a gel or regulate the activity of PMEs, coordinating the stiffening of the cell wall (Bosch et al., 2005; Bosch and Hepler, 2005; Parre and Geitmann, 2005; Tian et al., 2006; Vieira and Feijo', 2016). pH regulation of root cell elongation has been demonstrated directly (Fendrych et al., 2016). Thus, while never directly demonstrated, the regulation of the excretion of these ions to the apoplast could have a regulatory role in the anisotropy of cell wall mechanics of PTs.

ION FLUXES AND GRADIENTS AT THE TIP: AN ELECTRIFYING AFFAIR?

The particular constitution and regulation of the tip domain of the PT PM is such that large extracellular ion fluxes and cytosolic gradients are formed (Fig. 1). Ion fluxes, and in particular the huge anion efflux at the tip (Zonia et al., 2002; Gutermuth et al., 2013), are expected to generate an osmotic gradient, an extracellular electric field, and, as discussed below, eventually a membrane voltage gradient along the length of the PM. In Arabidopsis, the accumulation of vesicles at the apex is enough to sustain a 30-s growth period (Ketelaar et al., 2008). It has been proposed that vesicles in the clear zone are governed mainly by Brownian dynamics because of the apparent disorganization of the actin cytoskeleton in this region (Kroeger et al., 2009). But the existence of such dramatic ion gradients also may play a role in the movement of vesicles by either electrostatic

BOX 1. Taming turgor, the force that sustains growth: lessons from guard cells

PT growth is sustained by a minimum turgor (0.3 MPa in lily; Benkert et al., 1997). The relative importance of turgor and wall mechanics for growth is wrapped in controversy, (Winship et al., 2011; Zonia and Munnik, 2011), but consensus exists around turgor providing the force underlying wall elongation. Turgor implies the accumulation of solutes, namely ions. So how are they transported? And how do ions regulate water flux and turgor?

Guard cells are arguably the best studied cellular system of turgor regulation in plants, relying on H^+ , anions, and K^+ to move water in and out of the cell. Evidence for the same exists for PTs: H^+ fluxes are well documented (Feijó et al., 1999; Certal et al., 2008), and large anion fluxes of up to $60 \text{ nmol cm}^{-2} \text{ s}^{-1}$ (Zonia et al., 2002), anion conductivity (Tavares et al., 2011a), and a cytosolic anion gradient (Gutermuth et al., 2013) all have been characterized. Furthermore, K^+ currents and the underlying channels have long been observed in pollen (Obermeyer and Kolb, 1993; Mouline et al., 2002; Becker et al., 2004). So is it possible to establish a parallel between what is known about turgor regulation in guard cells and PT growth?

Similar events leading to the stomata **OPENING** are observable in pollen growth, with the efflux of H^+ in the **SHANK** coupling anion and K^+ influxes (Zonia et al., 2002; Domingos P, Dias PN, Tavares B, Portes MT, Konrad K, Wudick MW, Gilliam M, Bicho A, Feijó JA, unpublished data), which may indicate the water influx point. Conversely, as in stomatal **CLOSURE**, large effluxes of anions, and the influx of H^+ and Ca^{2+} , occur in the pollen **TIP**, the combined effect of which should result in the depolarization of the PM (Michard et al., 2009). We hypothesize that water flows at the pollen tip following the osmotic potential of anions (Cl^-/NO_3^-) and K^+ . This comparison implies that the mechanisms behind the temporal sequence of events in guard cells, leading to a transient water flow, could have been coopted in the PT, in the form of a spatial segregation of transport molecules between shank and tip, leading to the regulation of water transport and turgor necessary for growth (Fig. 3). This concept generates various testable hypotheses and gives ground to new channel discovery in PTs based on similarities with guard cells.

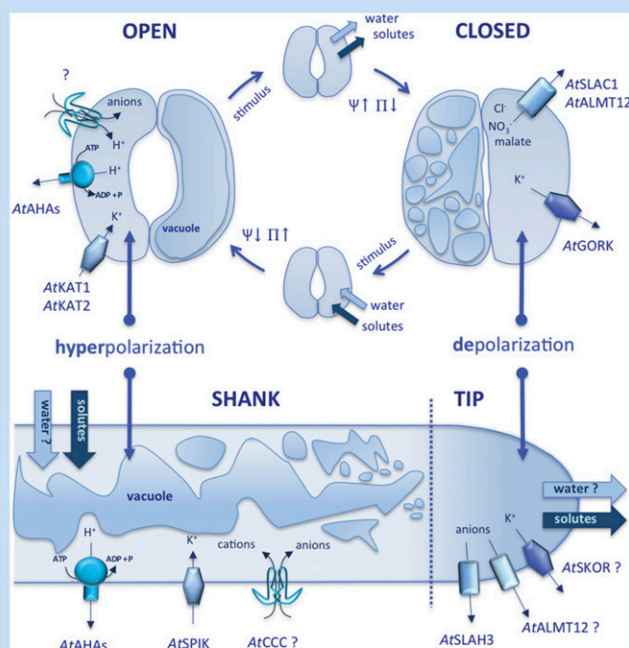


Figure 3. Schematic drawing illustrating the parallels between events leading to the regulation of guard cell (GC) movement (top) and putative mechanisms leading to PT water intake regulation and growth (bottom). Top, Stomatal opening and closing depend on GC turgor pressure regulation and are accompanied by morphological changes of the GC vacuoles. Stomatal opening (left) by stimuli such as light is sustained by solute influx that induces (1) water potential (Ψ) decrease, (2) turgor pressure (Π) increase, and (3) transient water influx. Main solute transporters are indicated in the scheme. H^+ pumps (AtAHA family) hyperpolarize the membrane and generate a H^+ gradient. K^+ enters through hyperpolarization-activated channels (mainly AtKAT1 and AtKAT2), and anions enter through an unidentified H^+ /anion cotransporter. During stomatal closure (right), in response to dark or abscisic acid, for instance, solute efflux induces (1) an increase in Ψ , (2) a decrease in Π , and (3) a transient water efflux. The anion channels AtSLAC1 and AtALMT12 catalyze massive anion effluxes that contribute to a depolarization of the PM. Potassium moves out of the cell through the depolarization-activated AtGORK channel. Bottom, The transport system at the shank of the PT is similar to the one involved in the opening of GCs: the activity of H^+ pumps (AtAHAs) couples the influx of K^+ and anions. The hyperpolarization-activated potassium channel involved (AtSPIK) is homologous to AtKAT1/2. The transport system at the growing tip can be compared with the one active in a closing GC: a large anion efflux is induced by the AtSLAH3 channel (homologous to AtSLAC1), and the expression of the depolarization-activated AtSKOR channel (homologous to AtGORK) in pollen may facilitate K^+ efflux. From this comparison of GC and PT, we propose testable hypotheses regarding membrane electric polarization along the PT as well as on water fluxes.

or osmotic influence. PM voltage has been determined to be around -130 mV when measured on the shank of PTs (Mouline et al., 2002; Griessner and Obermeyer, 2003; Becker et al., 2004), a resting potential slightly more negative than the K^+ equilibrium (Mouline et al., 2002). This hyperpolarization is likely driven by H^+ -ATPase activity (Lang et al., 2014). Unfortunately, the apical voltage remains unknown, since it is not possible to impale an electrode at the tip without provoking tube burst. Theoretically, apical membrane

potential can be inferred to be more positive compared with the shank, since large passive ion fluxes at the tip have a depolarizing effect. Furthermore, H^+ -ATPases are excluded from the tip (Certal et al., 2008; Michard et al., 2009), where there is high NADPH oxidase activity (Potocky et al., 2007). This kind of oxidase was proposed recently to generate electrochemical fluxes of ions on their own (Segal, 2016). Voltage at the tip PM and at the shank PM tend to equilibrate to the same value by charge fluxes along the membrane through the

equivalent of an electrical circuit, implying electrical resistance of the cytosol and capacitance of the membrane (Hille, 2001; Michard et al., 2009). Thus, a combination of high enough resistance of the cytosol and strong charge flux density across the PM at the tip could induce a membrane voltage gradient between the tip and the shank, supporting the notion of electrostatic movement of charges. Exocytic vesicles have long been found to bear a negative surface charge (Heslop-Harrison and Heslop-Harrison, 1982), and the existence of electrostatic fields in plant cells was recently proposed to underlie cell identity and signaling, namely by regulating the transfer of proteins from endomembrane to PM on the basis of charge alone (Simon et al., 2016). In animals, membrane depolarization promotes the vesicle exocytosis of pancreas β -cells (Yang et al., 2014; Cardenas and Marengo, 2016). If differential conditions of cytosolic resistance exist, then theoretically such mechanisms also could play a role in vesicle migration and fusion in PTs.

But other cues for polarity could come from anion fluxes. Large anion conductivity has been recorded in both *Arabidopsis* and tobacco (Tavares et al., 2011a, 2011b) with effluxes up to $60 \text{ nmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ (Zonia et al., 2002), resulting in an anion gradient (Gutermuth et al., 2013; Fig. 1B). The anion channel AtSLAH3 is partly responsible for this conductance (Gutermuth et al., 2013), but other anion channels expressed in the PT may be involved (Tavares et al., 2011a), like members from the AtALMT family (Meyer et al., 2010). The existence of a gradient and fluxes of anions of such magnitude has been proposed to be physically sufficient to create conditions for an osmotic gradient strong enough to generate thrust for vesicles to move toward a minimum osmotic potential at the apex through the process of osmophoresis (Lipchinsky, 2015). While proposed on theoretical grounds, the existence of biophysical mechanisms for the vectorial movement of vesicles in the clear zone is an exciting new prospect calling for experimental validation.

Although the direction of potassium fluxes in the tip is still debated, anion fluxes must be compensated by cation efflux (Michard et al., 2009). K^+ outward currents have been recorded by patch clamp (Griessner and Obermeyer, 2003) but remain unaccounted for in terms of the channel generating them. The SKOR K^+ channel is known to be expressed in pollen (Pina et al., 2005) and constitutes a good candidate for that function. Other channels, namely nonselective cationic channels, as well as transporters from the cation-proton antiporter family also could play a role in anion flux compensation (see below).

The Ca^{2+} Affair

A Ca^{2+} gradient in which intensity correlates with growth rate has long been described in PTs (Reiss and Herth, 1985; Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Pierson et al., 1994, 1996; Malho et al., 1995; Michard et al., 2008; Iwano et al., 2009; Fig. 1C). When

the tube is reversibly stopped by caffeine or low temperature, the Ca^{2+} gradient dissipates and the Ca^{2+} influx is lowered to a basal level; regrowth reconstitutes the Ca^{2+} gradient and influx (Pierson et al., 1996). Ca^{2+} channel activity was early deduced by quenching with Mn^{2+} (Malho et al., 1995). Several types of channels permeable to Ca^{2+} have been characterized by patch clamp either as inward rectifiers in *Arabidopsis* and pear (*Pyrus communis*; Wang et al., 2004; Shang et al., 2005; Wu et al., 2010, 2014) or with no clear rectification in tobacco (Michard et al., 2011). Among the 20 cyclic nucleotide-gated channels (CNGCs) in plants, at least CNGC7, CNGC8, CNGC16, and CNGC18 are expressed in pollen (Pina et al., 2005; Bock et al., 2006; Kaplan et al., 2007). Some CNGC channels have been presented as inward-rectifying channels when expressed in HEK cells or oocytes (Leng et al., 1999, 2002; Ma et al., 2007). The disruption of AtCNGC18, localized in the subapical membrane, induces male sterility due to defective PT growth (Frietsch et al., 2007). AtCNGC18 was recently shown to drive inward cationic currents activated by cAMP/cGMP in an animal heterologous system (Gao et al., 2016). AtCNGC7, which localizes at the flank of the growing tip, and AtCNGC8 have weaker phenotypes, but the double knockout is male sterile (Tunc-Ozdemir et al., 2013a). Lastly, AtCNGC16 only plays a role under stress conditions (Tunc-Ozdemir et al., 2013b). In animals, CNGCs have calmodulin (CaM)-binding domains with regulatory functions, a mechanism recently confirmed for AtCNGC12 in plants (DeFalco et al., 2016). In tobacco, the Glutamate receptor agonist D-Ser induces a Ca^{2+} current while the antagonist CNQX inhibits the Ca^{2+} conductivity of PT protoplasts (Michard et al., 2011). This finding led to the description of PT growth phenotypes for the *Arabidopsis* Glutamate receptor-like (GLR) mutants *glr1.2* and *glr3.7*. Additional GLRs also are expressed in PTs (Pina et al., 2005; Bock et al., 2006) and may be involved in Ca^{2+} homeostasis. In accordance, other plant GLRs have been localized on the PM, and AtGLR3.4 has been shown to induce Ca^{2+} accumulation in HEK cells (Meyerhoff et al., 2005; Tapken and Hollmann, 2008; Teardo et al., 2010; Vincill et al., 2013). Interestingly, GLRs heterodimerize, as demonstrated by the sensitivity profile to amino acids in knockout plants (Stephens et al., 2008), yeast two-hybrid analysis (Price et al., 2013; Vincill et al., 2013), or FRET (Vincill et al., 2013). Utilizing a chimera strategy by introducing the pore of plant AtGLR1.1 and AtGLR1.4 into the animal GluR1 channel demonstrated low Ca^{2+} permeability and low rectification with nonselective cation pores (Tapken and Hollmann, 2008). Other Ca^{2+} -permeable channels are putatively active in pollen on the basis of transcriptomics/proteomics, namely the annexins (Lee et al., 2004; Zhu et al., 2014) and the mechanosensitive channel AtOSCA1 (Yuan et al., 2014).

One common point of the Ca^{2+} -permeable channels identified so far is their weak selectivity: they appear to be nonselective cation channels rather than Ca^{2+} -selective channels. Their gating also is still poorly

defined. While CNGCs open in response to cAMP and cGMP in HEK cells, no data are available in situ. GLR channel activity is modulated by amino acids with low specificity, as shown by the different responses to Gly, Glu, and D-Ser (Michard et al., 2011). AtGLR1.4 and AtGLR3.4 induce nonselective cationic currents in response to a broad range of amino acids in mammalian cells (Tapken and Hollmann, 2008; Vincill et al., 2013; Tapken et al., 2013). Clearly, a common ligand-gated system for Ca^{2+} homeostasis is still missing in plant biology.

H⁺ Signaling, the Missing Link?

H⁺ has long been proposed to act in signaling cascades in plants (Felle, 2001) and has even been identified as a bona fide neurotransmitter (Beg et al., 2008). In PTs, H⁺ gradients also have been correlated with growth, with an acidic tip and alkaline subapical or submembranar region in tobacco and lily (Feijó et al., 1999; Certal et al., 2008; Michard et al., 2008). H⁺ influx at the apex and efflux at the shank have been recorded and participate in the establishment of the gradient (Feijó et al., 1999; Certal et al., 2008; Michard et al., 2008). This is particularly clear in lily, where an alkaline band in the subapical region coincides with a maximum proton efflux (Feijó et al., 1999). Interestingly, this pattern is different in tobacco. PTs from this species do not display a distinct alkaline band (Michard et al., 2008). Accordingly, the H⁺ effluxes along tobacco tubes do not display a maximum in the subapical zone as they do in lily but look rather constant along the tube (Certal et al., 2008). To the best of our knowledge, no H⁺ or H⁺-permeable channels have been characterized in plants. Anion channels have been discussed as participating in the establishment of pH gradients (Ferne and Martinoia, 2009). In addition to the endogenous permeability of the membrane bilayer to H⁺ (Gutknecht, 1987), H⁺ symporters or antiporters may be involved in H⁺ fluxes. PTs express 18 genes from the putative cation-H⁺ antiporter CHX family, with at least six genes expressed in the vegetative cell (Sze et al., 2004). Recently, CHX19 was localized in pollen PM (Padmanaban et al., 2017), while other GFP chimeras usually localize to the endomembrane system (see below; Table I).

THE SHANK OF THE PT: JUST SUPPORTING AND BUFFERING?

H⁺ (pH) and Ca^{2+} gradients seem to depend on at least three components to be established: (1) influxes at the tip; (2) a cytoplasmic component including buffering by proteins and endomembrane transport activity; and (3) efflux at the shank. This section deals with the latter two.

The PT membrane appears to play an important role in the gradient establishment by its H⁺- and Ca^{2+} -ATPase activity. PM H⁺-ATPases play a major role in pH

regulation (Sanders et al., 1981). The tobacco proton pump, NtAHA1, is specifically excluded from the PT tip, as shown by a GFP fusion (Certal et al., 2008). Early studies demonstrated that the inhibition of Ca^{2+} pumps using vanadate and compound 48/80 induces the increase of intracellular Ca^{2+} (Obermeyer and Weisenseel, 1991). Pharmacological studies on a Ca^{2+} -ATPase reconstituted in proteoliposomes showed that it can act as a bona fide $\text{Ca}^{2+}/\text{H}^+$ exchanger (Luoni et al., 2000). The fact that in PTs there is a high tip Ca^{2+} concentration and a low pH also could be dependent, at least in part, on the activity of this pump. The calcium pump AtACA9 is expressed specifically in pollen, and the knockout plant of this transporter displays a defect in pollen growth (Schlott et al., 2004). A major role is suspected to be played by 14-3-3 proteins due to their regulation of the C-terminal activity of all H⁺-ATPases, and their proteomics repertoire has been described (Pertl et al., 2011). Yet, the large diversity and the lack of genetic studies still hinder their true mechanistic impact on the pH regulation of PTs.

H⁺ and Ca^{2+} are highly buffered in the cytoplasm. pH homeostasis is largely ensured by metabolic regulation through the classical pH-STAT pathway (Smith and Raven, 1979; Sakano, 2001). Ca^{2+} interacts with many proteins in the cytosol that lower its diffusion coefficient (White and Broadley, 2003). In addition, H⁺ and Ca^{2+} fluxes from and into internal stores are accepted to play a major role in the dynamics of those ions inside the cell, but there are no quantifications or mechanisms described. While the role of H⁺ and Ca^{2+} influxes at the tip in generating H⁺ and Ca^{2+} gradients is well defined, the role of cytosol and endomembrane proteins that are possibly major players in shaping the gradients is only vaguely known.

Several PM transporters are expressed in apical vesicles, including the P-type H⁺-ATPases that may participate in local pH regulation (Certal et al., 2008). These H⁺ pumps were recently associated with the rapid alkalization factor (RALF) and the important receptor-like kinase FERONIA in roots (Haruta et al., 2014). Pollen expresses a number of RALFs (Pina et al., 2005), and a role of RALFs in PT growth regulation has long been described (Covey et al., 2010). Given that both FERONIA and its male counterpart ANXUR have disruptive fertilization phenotypes (for review, see Li et al., 2016), this functional relationship between RALFs and the H⁺-ATPases could have consequences for pH regulation in PTs as well.

Cation-H⁺ antiporters, from the CPA1 and CPA2 family, may play a role in controlling intracellular pH. CPA1 is typically localized in endomembranes, and some of them are expressed in pollen (Sze et al., 2004). From this family, Na⁺(CATION)/H⁺ EXCHANGER (AtNHX1) and AtNHX2 control cell expansion, pH homeostasis, and K⁺ accumulation in the vacuole (Bassil et al., 2011). AtCHXs are expected to modulate pH by catalyzing H⁺- and K⁺-coupled fluxes. AtCHX13 and AtCHX14, both expressed in pollen, are PM transporters involved in K⁺ homeostasis (Sze et al.,

2004; Zhao et al., 2008, 2015). CHX13 is a high-affinity K^+ influx transporter (Zhao et al., 2008), while AtCHX14 is a low-affinity efflux K^+ transporter in yeast and plant cells that operates in a pH-dependent manner (Zhao et al., 2008, 2015). Other AtCHXs, notably AtCHX21 and AtCHX23, have been characterized to play a role in pollen navigation through the ovary, resulting in male-transmitted sterility of the double knockout *chx21/23*, although PTs grow normally in vitro and in vivo (Evans et al., 2011; Lu et al., 2011). AtCHX23 increases yeast tolerance to high potassium in a pH-dependent manner, suggesting that it is an H^+/K^+ antiporter, and recent structure-function studies on AtCHX17 opened the way for understanding the integrated physiological function of this pollen overrepresented transporter family (Czerny et al., 2016). Recently, *CHX17* and *CHX19* were localized in PT vegetative and sperm cells, respectively, *CHX19* being addressed to the PM (Padmanaban et al., 2017). The triple mutant displays a fertility phenotype as well as a compromised pollen cell wall formation that could affect germination, tube burst, as well as gamete function (Padmanaban et al., 2017).

Ca^{2+} internal stores may also be essential in the establishment of the calcium gradient. In the absence of the canonical ligand-operated Ca^{2+} -buffering mechanisms as described for animal cells, namely IP₃ receptors and G-coupled receptors, the repertoire of possible candidates in plants include the CAX (cation- H^+ exchangers) and TPC (two-pore channels) families. TPC was recently involved in the propagation of Ca^{2+} signals in roots (Choi et al., 2014), but no male phenotypes have been described. Measurement of Ca^{2+} in the endoplasmic reticulum (ER) showed that an inhibition of growth in parallel with a decrease in ER Ca^{2+} occurred when the ER Ca^{2+} -ATPase was inhibited (Iwano et al., 2009). Some GLRs are expressed in endomembranes and may participate in Ca^{2+} signaling (Teardo et al., 2011, 2015). Experimental evidence also is suggestive of a role for the Arabidopsis mitochondrial channel uniporter regulator MICU, a Ca^{2+} -binding protein that modulates the mitochondrial Ca^{2+} accumulation (Stael et al., 2012). Interestingly, MICU is expressed in PTs (Wang et al., 2008b). Mitochondrial membrane charge shifts at the clear zone of PTs (Colaço et al., 2012) have been speculated to play a role in cytosolic Ca^{2+} homeostasis and in the definition of the Ca^{2+} gradient at the tip.

Reactive oxygen species (ROS) also have been long implicated in ion dynamics regulation in PTs. The subject has been reviewed elsewhere (Wudick and Feijó, 2014; Mangano et al., 2016), so it will only be alluded to briefly here. Tip-localized ROS in growing PTs have been described in Arabidopsis (Potocky et al., 2007), and double mutants of the pollen-expressed H and J members of respiratory burst oxidase homolog (Rboh) have PT phenotypes and altered Ca^{2+} gradient features (Liu et al., 2009; Boisson-Dernier et al., 2013; Kaya et al., 2014, 2015; Lassig et al., 2014). Plastids and mitochondria positioned in the subapical region are other possible sources of ROS (Mittler et al., 2011). In lily, abundant accumulations of ROS have been linked

to mitochondria (Cardenas et al., 2006). This competing view of tip-localized ROS raises questions about ROS concentrations in the shank as either free cytosolic or localized to subcellular vesicles. Hydrogen peroxide was recently implicated in the regulation of K^+ and Ca^{2+} conductivities in lily pollen protoplast (Breygina et al., 2016). The possible integration of these pathways is illustrated in Text Box 2 (Zhou et al., 2014; Zhao et al., 2013; Xu et al., 2006; Mahs et al. 2013; Kaya et al., 2015; Garcia-Mata et al., 2010).

DECODING THE ION CODE: DOWNSTREAM MECHANISMS OF INTEGRATION OF ION SIGNALING

Various Ca^{2+} sensor proteins are known, some of them with activity in PTs. The most prominent are within the Ca^{2+} -binding EF-hand superfamily, which consists of calmodulin, calmodulin-like (CML) and calcineurin B-like (CBL) proteins, CBL-interacting protein kinases (CIPK), and finally the Ca^{2+} -dependent protein kinases (CDPK; abbreviated CPK in Arabidopsis; Zhou et al., 2015a). CaM, CML, and CBL have been demonstrated previously to serve as sensor relays that lack enzymatic activity, while CIPK and CDPK are responders. Each of these proteins contains a special helix-loop-helix motif known as the EF-hand that enables Ca^{2+} binding (Konrad et al., 2011; Steinhorst and Kudla, 2013).

The CPK family appears to have the most diverse array of targets by interacting with actin and also other membrane proteins (Curran et al., 2011). Of the 34 CPKs present in the genome of Arabidopsis, 12 members (CPK2, CPK4, CPK6, CPK11, CPK14, CPK16, CPK17, CPK20, CPK24, CPK26, CPK32, and CPK34) are expressed in pollen (Honys and Twell, 2003; Hrabak et al., 2003; Harper et al., 2004; Pina et al., 2005). Five of these have been characterized: AtCPK17 and AtCPK34 show very strong PT growth phenotypes; therefore, these proteins must play important roles, which were hypothesized to regulate Ca^{2+} channel activity (Myers et al., 2009). The expression of the 12 pollen Arabidopsis CPKs was analyzed by transient expression in tobacco PTs, revealing differential localizations: CPK4, CPK11, and CPK26 are cytosolic; CPK16, CPK24, and CPK32 seem to localize preferentially in the generative cell membrane; while the others localize to the PM of the PT: CPK2 and CPK20 specifically at the tip, CPK17 and CPK34 in the subapical zone (Gutermuth et al., 2013). Furthermore, AtCPK2 and AtCPK20 were shown to interact and activate the conductivity of the anion channel AtSLAH3. Taken together with the counter-correlation between the levels of cytosolic Ca^{2+} and Cl^- , these data are suggestive of a feedback regulation loop that could underlie the control of growth as an interplay between these two ions, AtSLAH3 and a yet unknown depolarization-activated Ca^{2+} channel (Gutermuth et al., 2013).

CMLs sense free cytosolic Ca^{2+} and are likely integral targets of Ca^{2+} signaling. There are over 50 AtCMLs expressed within pollen that share the highly

BOX 2. Integrating pathways: mission impossible?

All things are connected, and any exercise of linking different pathways is perennial by nature but often useful to develop hypotheses on their functional interfaces. Figure 4 represents one such exercise based on the available data. Ca^{2+} is known to activate AtCPK32, which in turn activates the Ca^{2+} -permeable channel AtCNGC18; overexpression of AtCPK32 also produces Ca^{2+} accumulation and unpolarized growth (Zhou et al., 2014). AtCPK11 and AtCPK24 inhibit inward K^+ currents via the Shaker channel AtSPIK (Zhao et al., 2013). AtCPK2 and AtCPK20 activate the anion channel AtSLAH3, and this interaction is suggestive of a growth regulatory feedback loop involving a yet uncharacterized depolarization-activated Ca^{2+} channel (Gutermuth et al., 2013). Overexpression of AtCIPK19 led to increased apical Ca^{2+} and loss of polarity, whereas the loss-of-function mutant hindered growth (Zhou et al., 2015a). In root hairs, CBL1 and CBL9 were shown to interact with CIPK23 to target the Shaker-type K^+ channel AKT1 (Xu et al., 2006). In PTs, Mahs et al. (2013) identified a similar role for CBL1 and CBL9 as Ca^{2+} sensors that show overexpression and knockout phenotypes of disturbed K^+ homeostasis, leading to the speculation that CBL1 and CBL9 may interact with an unknown CIPK to regulate a Shaker-type K^+ channel homologous to AKT1, SPIK. In PTs, AtCBL2 and AtCBL3 interact with AtCIPK12 on the tonoplast, implicating the vacuole in Ca^{2+} homeostasis and signaling (Steinhorst et al., 2015). CaM and CPKs have been identified as interacting partners to enable H^+ -ATPases (Pertl-Obermeyer et al., 2014). ROS regulate Ca^{2+} influxes through Ca^{2+} -permeable channels in root hairs, while in PTs, Ca^{2+} has been shown to regulate the activity of the ROS-producing proteins AtRbohH and AtRbohJ (Wu et al., 2010; Kaya et al., 2015; for review, see Wudick and Feijó, 2014). The outward K^+ channel AtSKOR responds to hydrogen peroxide (García-Mata et al., 2010). In PT experiments in which AtRbohH and AtRbohJ activity was manipulated, AtSPIK and AtSKOR have been suggested to be regulated by ROS (Lassig et al., 2014). Cytosolic pH affects the activity of many proteins, yet specific regulation of tube PM transporters by pH has not been reported so far. Given the existence of defined gradients of acidity on the tip and alkalinity either cytoplasmic or submembranar (Fig. 1), pH could regulate differentially evenly distributed channels or be involved in processes to regulate the differential localization of channels, one or the other contributing to the stabilization of cell polarity at the tip.

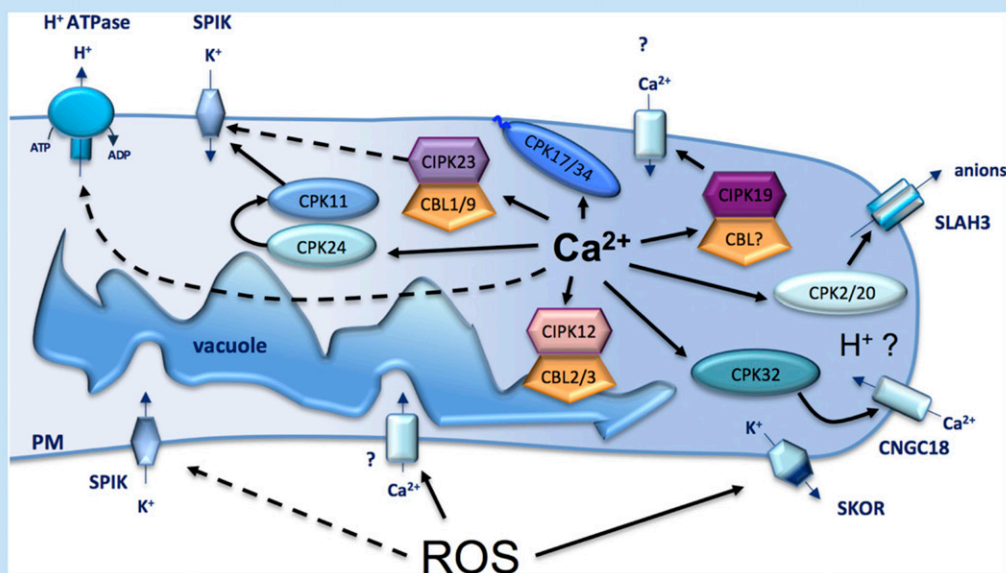


Figure 4. Schematic overview of signaling networks based on ion secondary messengers that participate in establishing ion homeostasis. Solid lines represent supported findings, while dashed lines represent ongoing hypotheses. Question marks indicate the likely presence of proteins whose molecular identities are still to be determined. We have represented the main transporters here: proton pumps, inward K^+ channel AtSPIK on the tube, calcium channels (unknown and AtCNGC18), outward potassium channel AtSKOR, and AtSLAH3 anion channel on the tip (for details, see Fig. 2; Table I).

conserved EF-hand binding motif and up to 75% amino acid identity with CaM (Bender and Snedden, 2013). Pharmacological evidence of the action of CaM in PTs of *Arabidopsis* seems to implicate hyperpolarization-activated Ca^{2+} channels, suggestive of a closed feedback loop dependent on H^+ -ATPase activity (Sun et al., 2009). More recently, a function was identified for AtCML24 in PT growth, acting on the interface between the actin cytoskeleton and Ca^{2+}

(Yang et al., 2014). Given the relevance of AtCNGCs in PT growth, the recent description of CaM regulation of AtCNGC12 (DeFalco et al., 2016) opens the possibility that such a protein also may also be involved as a decoder of Ca^{2+} signals in PTs, as previously suggested by injection experiments (Moutinho et al., 1998; Rato et al., 2004).

CIPKs are Ser/Thr kinases that couple with CBLs in the Ca^{2+} signaling network (Edel and Kudla, 2015; Mao

Table II. Cytoskeleton effects linked to Ca²⁺, H⁺, or ROS signaling

Second Messenger	Downstream Target(s)	Cytoskeleton Effects (<i>species</i>)	References
Ca ²⁺	<p>ROP1 → RIC1 ROP1 → RIC3 ROP1 → RIC4</p>	<ol style="list-style-type: none"> 1. F-actin severing 2. Capping barbed ends (<i>At</i>) <p>F-actin disassembly</p> <ol style="list-style-type: none"> 1. F-actin assembly 2. Increases exocytosis of vesicles (<i>Nt</i>, <i>At</i>) 	Zhou <i>et al.</i> , 2015; Gu <i>et al.</i> , 2005
	AtMDP25	F-actin severing (<i>At</i>)	Qin <i>et al.</i> , 2014
	AtMAP18	F-actin severing (<i>At</i>)	Zhu <i>et al.</i> , 2013
	Myosin XI?	Low Ca ²⁺ (-0.1μM) generates cytoplasmic streaming. Near 1μM Ca ²⁺ , streaming ability inhibited (<i>Ll</i>).	Yokota <i>et al.</i> , 1999; Tominaga <i>et al.</i> , 2012
	Profilin	<ol style="list-style-type: none"> 1. Increased Ca²⁺ results in increased depolymerization (<i>Zm</i>, <i>Pr</i>) 2. Increasing Ca²⁺ sequesters profilin to block function (<i>Zm</i>, <i>Pr</i>) 	Kovar <i>et al.</i> , 2000 Snowman <i>et al.</i> , 2002
	Villin2, 5?	Present in apical and sub-apical region. Actin severing, collar formation. (<i>At</i>)	Qu <i>et al.</i> , 2013
	<i>Ll</i> ABP29	Binds and aids F-actin severing. Overexpression inhibited germination and tube growth (<i>Ll</i>)	Xiang <i>et al.</i> , 2007
	<i>Ld</i> ABP41	F-actin severing (<i>Ld</i>)	Fan <i>et al.</i> , 2004
	<i>Pr</i> ABP80 (gelsolin-like)	F-actin severing, barbed end capping (<i>Pr</i>)	Huang <i>et al.</i> , 2004
H ⁺	<i>Ll</i> LIM1	F-actin bundle assembly and stabilization (<i>Ll</i>)	Wang <i>et al.</i> , 2008
	Actin-Depolymerizing Factor (ADF) and Actin-Interacting Protein (AIP)?	Active at alkaline pH to promote actin depolymerization (<i>Nt</i>). AIP proposed to work cooperatively with ADF (<i>Ll</i>).	Chen <i>et al.</i> , 2002; Lovy-Wheeler <i>et al.</i> , 2006
ROS	Ca ²⁺ → CIPK → ?	Phosphorylation of actin depolymerizing agents? No evidence ROS acts directly on cytoskeleton.	Chen <i>et al.</i> , 2002; Wudick <i>et al.</i> , 2014

et al., 2016). The AtCBL-AtCIPK complex has been heavily implicated in abiotic stress tolerance by adjusting the levels of K⁺ through AtAKT1 (Manik et al., 2015). Of the 26 different CIPKs identified within the Arabidopsis genome, AtCIPK10, AtCIPK11, AtCIPK12, AtCIPK14, and AtCIPK19 are strongly expressed in PTs (Konrad et al., 2011; Zhou et al., 2015a). They display an even distribution throughout the cytosol while being weakly expressed in other tissues (Zhou et al., 2015a). Different AtCBL/AtCIPK pairs have been implicated in the activation of various kinds of channels (for review, see Steinhorst and Kudla, 2013), and in PTs, AtCBL2 and AtCBL3 were described to interact with AtCIPK12 in the tonoplast, with phenotypic consequences at the level of growth and PT morphogenesis, implicating the vacuole in Ca²⁺ homeostasis and signaling (Steinhorst

et al., 2015). A possible integration of these pathways is offered in Figure 4.

CYTOSKELETON AND ION DYNAMICS

PT growth is accredited to actin polymerization (Vidali and Hepler, 2001; Cardenas et al., 2008). Yet, a collective look at actin dynamics suggests an alternative perspective for normal growth: it is rapid actin turnover, the continuous assembly and disassembly of actin, that is important variable. Are signaling gradients, and not strict concentrations, important by themselves through dynamic mechanisms such as the polymerization/depolymerization of actin? Possible interactions between H⁺ and Ca²⁺ and actin related

proteins have been systematized previously (Feijó et al., 2004; Hepler, 2016) and are summarized in Table II. One prominent candidate for interaction with Ca^{2+} is the GTPase Rho family of plants (ROPs). Primarily tip localized, members such as the pollen-specific AtROP1 have been demonstrated to mediate F-actin and Ca^{2+} signaling (for review, see Cheung and Wu, 2008; Qin and Yang, 2011). The best characterized ROP downstream effector are the family of ROP-interactive CRIB-containing proteins (RICs) that show a diverse range of responses. Opposing pathways of AtRIC3 and AtRIC4 produced starkly different consequences on F-actin. AtRIC3 leads to F-actin disassembly, specifically at the tip of PTs, promoting the accumulation of tip-localized Ca^{2+} . Conversely, AtRIC4 in response to Ca^{2+} promotes F-actin assembly (Gu et al., 2005). Additionally, recent studies investigating the apical PM-localized AtRIC1 add to the importance of RIC to regulate actin (Zhou et al., 2015b). Similar to AtRIC3, AtRIC1 binds to and severs F-actin in the presence of Ca^{2+} . The *ric1* knockout mutant was characterized by an accumulation of actin at the fringe and increased PT growth rate (Zhou et al., 2015b). Despite multiple pieces of evidence and phenotypes, a consensual mechanism for ROP GTPases in PT growth is still debated.

A number of actin-binding proteins, such as LIABP29 and LdABP41, respond in a Ca^{2+} -sensitive manner to contribute to F-actin regulation (Fan et al., 2004; Xiang et al., 2007). Also, villins, which are known to be Ca^{2+} responsive (Yokota et al., 2005), have been found to be major players in the actin turnover in PTs (Qu et al., 2013). Further details of ABPs have been reviewed recently (Fu, 2015; Hepler, 2016). Of note, there are no reports of ion regulatory dependencies for the ABP family of formins, which have emerged as major regulators of actin organization in PTs (Cheung et al., 2010). Like all proteins, formin action should be dependent on pH, but the range of pH variations in the PT tip was never associated with their regulation.

Despite contradictory evidence for a crucial role of microtubules in PTs, microtubule-destabilizing protein (MDP) binding activity was found to be augmented by high Ca^{2+} in Arabidopsis. Yet, remarkably, its action seems to be to sever actin instead of tubulin. AtMDP25 is localized in the subapical region on the PM, corresponding to the highest concentrations of Ca^{2+} , where it directly binds to and severs actin filaments, and its knockout resulted in an increased PT growth rate but, paradoxically, reduced fertilization (Qin et al., 2014). Moreover, MICROTUBULE-ASSOCIATED PROTEIN18 demonstrates similar actin-severing functions, as it is also localized on the subapical PM (Zhu et al., 2013). At this point, it is difficult to develop a mechanism integrating actin and microtubule interaction, but some evidence exists that they may cooperate in order to regulate the mechanical properties of PTs (Gossot and Geitmann, 2007).

H^+ also may be involved in the cytoskeleton and exocytosis regulation. Of relevance, alkalization or

acidification treatment of growing PTs showed that pH may control actin dynamics in lily PTs, eventually through the ADF-cofilin complex (Lovy-Wheeler et al., 2006). Other evidence of H^+ targeting the cytoskeleton comes from studies on the actin-binding protein LILIM1, which is essential for F-actin bundle assembly and protection against latruncalin B-mediated depolymerization and exhibits preference for activity under low pH and low calcium concentrations (Wang et al., 2008a).

CONCLUSION

PTs have been the source of relevant information of channel identity and function, mostly due to their strict dependence on ion dynamics and favorable cell biology. The aspects covered in this review show not

OUTSTANDING QUESTIONS

- What are the channels and transporters that sustain the cytosolic gradients of Ca^{2+} and anions in PTs?
- Are specific choreographies of ion dynamics associated with the spatial and temporal coordination of cellular processes that lead to growth and morphogenesis of the pollen tube?
- How are ions, especially K^+ and anions, associated with the generation of turgor and regulation of wall synthesis?
- What is the contribution of the endomembrane system and vacuole to ion homeostasis in the cytosol? And how are the ion transports to these compartments regulated?
- Is there a ligand-based regulatory mechanism that coordinates the activity of ligand-gated channels for the regulation of ion cytosolic concentration, especially for Ca^{2+} ?
- What are the targets of the cytosolic pH gradient at the tip?
- How do the multiple downstream target families of Ca^{2+} (CPKs, CIPKs, and CMLs) interact to produce specific responses?

only significant advances in the repertoire of known ion transport proteins but also significant advances in downstream regulatory targets that may elucidate the very fundamental levels of organization of a living cell. Also in that respect, PTs offer unique features to bridge to biophysics, specifically at the levels of electrochemistry and biomechanics. Most of the studies covered refer to in vitro PT growth phenotypes, eventually backed up by in vivo phenotyping, but several pieces of evidence suggest that ion dynamics mechanisms can actually be involved in various steps of the progamic phase of reproduction. Examples of this centrality come from recent demonstrations of the influence of pollen Ca^{2+} -ATPases (Iwano et al., 2014) and GLRs (Iwano et al., 2015) on the self-incompatibility of *Brassica* spp. and the suite of articles that defined the emergence of an ion-mediated signaling model during PT-embryo sac interaction, either through K^+ in maize (*Zea mays*; Amien et al., 2010) or the putative existence of Ca^{2+} signatures in Arabidopsis (Iwano et al., 2012; Denninger et al., 2014; Hamamura et al., 2014; Ngo et al., 2014). The context of cell-cell communication during reproduction is suggestive that PTs have evolved for fast reaction signaling in the form of chemotaxis, growth turns, growth rate alterations, and bursting; therefore, it is likely that the repertoire of basic mechanisms that transduce information through ion dynamics will be increased, benefited by the plethora of new imaging methods and genetic probes continuously being developed.

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