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Review

Calcium - a central regulator of pollen germination and tube growth ☆

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ABSTRACT

Pollen tubes grow rapidly by very fast rates and reach extended lengths to bring about fertilization during plant reproduction. The pollen tube grows exclusively at its tip. Fundamental for such local, tip-focused growth are the presence of internal gradients and transmembrane fluxes of ions. Consequently, vegetative pollen tube cells are an excellent single cell model system to investigate cell biological processes of vesicle transport, cytoskeleton reorganization and regulation of ion transport. The second messenger Ca^{2+} has emerged as a central and crucial modulator that not only regulates but also integrates the coordination each of these processes. In this review we reflect on recent advances in our understanding of the mechanisms of Ca^{2+} function in pollen tube growth, focusing on its role in basic cellular processes such as control of cell growth, vesicular transport and intracellular signaling by localized gradients of second messengers. In particular we discuss new insights into the identity and role of Ca^{2+} conductive ion channels and present experimental addressable hypotheses about their regulation. This article is part of a Special Issue entitled:12th European Symposium on Calcium.

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1. Introduction

During sexual reproduction of plants, germination of the male gametophyte (the pollen grain) and proper elongation of the growing tube are essential processes. A plethora of different activities and signals are involved in guiding the male sperm cells to their target, the haploid egg cell (the female gametophyte). In angiosperms after landing on the stigma the desiccated pollen grain rapidly rehydrates and starts to germinate. Subsequently, a pollen tube is growing out of the grain, penetrating the stigma tissue and starts to grow obliviously towards the ovule [1]. To find the right way to the ovule pollen tubes have to perceive and integrate guiding signals from the female tissue. However, even without such external guiding signals pollen grains are able to germinate and establish a polar growing tube in vitro due to a “self-organizing signaling network” [2]. This is one feature that renders this tip-growing cell an excellent model system and tool for investigation of various polar growth processes by quantification of germination rates and tube elongation. Remarkably, pollen tubes can elongate with a tremendous speed. Such pollen tubes growth rates have been reported to range from 1000 μm/h for lily pollen [2–5] and can reach up to 14400 μm/h in fast growing species like Tradescantia or Hemerocallis [6]. This is considerably faster than described growth rates of neuronal axons, which grow at a speed comparable to that of root hairs (~20–50 μm/h) [7,8]. Another remarkable feature of growing pollen tubes is the distance they cover. To deliver the sperm cells to the ovule, pollen tubes have to transcend distances like a few hundred micrometers in Arabidopsis and up to 50 cm in maize [6,9]. Expansion of the growing pollen tube exclusively occurs at the extreme apex. This exclusive apical growth results from coordinated changes in cell wall properties, endo- and exocytosis, cytoskeletal regulation, changes in ion concentrations and possibly subsequent changes in turgor pressure. Hypotheses that consider the prevalence of either a “cell wall model” or a “hydrodynamic model” as driving forces for pollen tube growth have recently been controversially discussed [10,11]. It still appears to represent an open question whether adjustments of turgor pressure take place in order to promote growth and so far no changes in turgor pressure in growing pollen tubes have been observed [11,12]. However, a contribution of the existing turgor pressure in the cell to its elongation appears conceivable. Complex interwoven networks of signaling events regulating the above mentioned components have evolved and are currently intensely investigated. Several signaling molecules function in these signaling networks during pollen germination and tube growth. Ca^{2+} appears to be involved in most if not in all of these processes. In the 1960s Brewbaker and Kwack were the first to describe the necessity of Ca^{2+} for pollen germination and pollen tube growth [13]. In the 1990s significant advancements have been made in characterizing and measuring the Ca^{2+} gradient within the tube and ion influxes from the extracellular space by Ca^{2+} sensitive dyes and ion selective vibrating electrodes [14–17]. Nowadays the importance of the tip-focused Ca^{2+} gradient and apical

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influx of this ion, as well as its contribution to polar growth of the tube are firmly established [4,18–21]. Functions that the second messenger ion Ca^{2+} fulfills in pollen range from a key role in the prevention of self-fertilization [22,23], regulation of the cytoskeleton via actin binding proteins [24–26], influence on vesicle dynamics and membrane trafficking [27,28] to its role as a constituent of the cell wall [29]. In this review we discuss the various functions of Ca^{2+} in the initiation and regulation of pollen germination as well as during the polar growth of the tube.

2. A tip-focused calcium gradient is essential for pollen germination and tube growth

In 1975 Jaffe et al. described patterns of Ca^{2+} accumulations and fluxes in growing pollen tubes of *L. longiflorum* which formed a tip-focused gradient and required an influx at the tip [30]. Today it is well established that in all species that have been studied the cytosolic concentration of free Ca^{2+} is highest in close proximity to the plasma membrane at the tip of the growing pollen tube [6,31–35]. The cytoplasmic Ca^{2+} concentration ranges from 2–10 μM in the apical 20 μm of the tip region to 20–200 nm in the shank of the tube. In contrast extracellular Ca^{2+} concentrations vary in a range of 10–10000 μM. Apical influx of Ca^{2+} ions from the extracellular space represents the predominant source establishing this gradient in combination with subapical secretion of this ion to internal stores and/or across the plasma membrane (Fig. 1) [14,16–18,36–40]. The necessity of this steep Ca^{2+} gradient for growth has been shown in the 1990s by application of Ca^{2+} channel blockers (e.g. La^{3+}) or injection of 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) buffer which inhibits elongation and at the same time abrogates the Ca^{2+} gradient [14,16–18]. Interestingly, the described cytosolic Ca^{2+} gradient is not only necessary for elongation of the tube. Additionally, it impacts on growth direction and alteration of its focal point leads to reorientation of the growth axis toward the site of the higher Ca^{2+} concentration [19]. The establishment of the growth directing Ca^{2+} gradient already begins in the rehydrated pollen grain where it defines the site where the tube will eventually protrude [33]. Treatment with the Ca^{2+} channel blocker nifedipine prevents establishment of the gradient, as well as germination [33,41]. Experiments with latrunculin B (Lat-B), which prevents actin polymerization, furthermore revealed a connection between actin dynamics and the Ca^{2+} gradient in lily pollen [42]. Application of Lat-B to growing tubes leads to growth retardation, followed by growth arrest and simultaneously the Ca^{2+} gradient dissipates. Besides this essential Ca^{2+} gradient there are also other ionic gradients existing in a growing pollen tube such as K^+, Cl^- and H^+. The latter gradient creates a constitutive alkaline band at the base of the clear zone, which contains mainly vesicles but no large organelles, and an acidic tip [43]. Although three studies suggest that pH gradients are not required or associated with tip growth in *Lilium longiflorum* or *Agapanthus umbellatus* pollen tubes [44–46], subsequent studies with *Lilium longiflorum* and tobacco pollen tubes led to the assumption that proton gradients exist and that they may be associated with polarized pollen tube growth [32,43,47]. Also tightly regulated fluxes of anions like most notably Cl^- appear to be crucial for pollen germination and pollen tube growth. This not very well understood aspect of pollen biology has been comprehensively reviewed and discussed in an excellent recent review [48]. Interestingly, the chloride anion has a reversed direction of flow compared to cations as it was observed to leak out at the tip and enter the tube at the shank [49]. The essential requirement of efficient K^+ transport into the growing pollen tube has been corroborated by analyses of the plasma membrane inwardly rectifying K^+ channel SPIK of the Shaker family. The respective *spik* mutant revealed an impairment of pollen tube growth [9]. Simultaneous modeling of diverse ion fluxes in growing pollen tubes based on all currently available experimental evidence supported a model for pollen electric polarization establishment and a role of transport system polarization in ion flux regulation (discussed in detail in [6]).

3. Calcium oscillations and tube growth

The above mentioned growth rates of pollen tubes represent peak values. In fact growth rates of elongating pollen tubes oscillate in many species. And so does the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) as virtually all growth related processes in the growing pollen tube oscillate [4,36]. $[Ca^{2+}]_{cyt}$ and the growth rate synchronously oscillate with amplitudes of 3–4 fold changes. Both oscillate with the same period (15–60 s) and in phase. Oscillations of $[Ca^{2+}]_{cyt}$ lag 10–40° behind growth rate, whereas influx of Ca^{2+} lags 11 s behind the growth peak in *Lilium longiflorum*. This corresponds to a phase shift of ~140° relating to a period of ~30 s [50]. Measurements that confirmed the latter finding were performed with an ion-selective vibrating electrode, which does not directly measure ion fluxes across the plasma membrane but fluxes from the extracellular space into the tube. These observations led to the

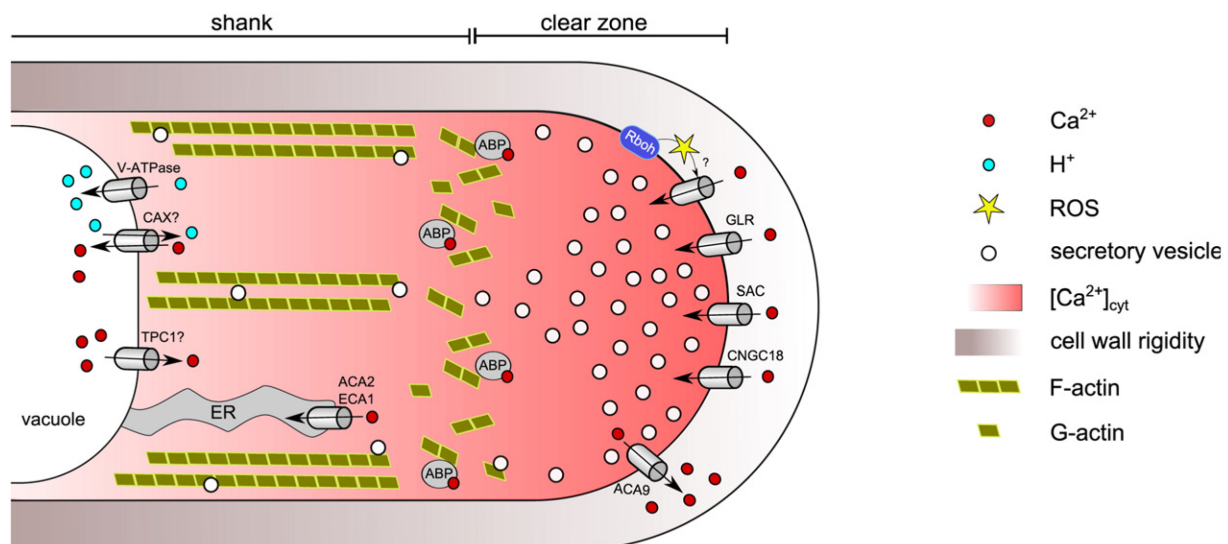

Fig. 1. Ca^{2+} regulated processes and changes in Ca^{2+} concentration in a growing pollen tube. This schema illustrates the diverse Ca^{2+} fluxes and concentration as well as their impact on actin organization in the pollen tube. See main text for further details.

hypothesis that the cell wall could serve as a storage compartment for Ca^{2+} ions [50]. In animal cells the divergence of increase in $[Ca^{2+}]_{cyt}$ and measured external flux could be explained by direct uptake of Ca^{2+} into the endoplasmatic reticulum (ER), the so called capacitive Ca^{2+} entry (CCE) [50,51]. However, studies reported by Malhó disproved this theory for the growing pollen tube [52]. Uptake of Ca^{2+} into the ER could rather be considered as a fine-tuning mechanism for Ca^{2+} oscillations in pollen tubes [34]. This uptake is likely to be accomplished by P-type II Ca^{2+}-ATPases, as suggested by the application of cyclopiazonic acid, an inhibitor of ER-type Ca^{2+}-ATPases, that inhibited pollen tube growth and decreased the $[Ca^{2+}]_{ER}$ (Fig. 1) [34]. Influx of Ca^{2+} ions from the external medium into the cytosol most likely involves the function of stretch-activated Ca^{2+} channels (SACs) at the extreme apex [53]. During phases of fast growth these channels are more likely to be open due to stretching of the plasma membrane, especially at the expanding tip (Fig. 1). However, the molecular identity of these channels has so far remained unknown. In contrast, a contribution of cyclic nucleotide gated channels (CNGCs) to the modulation of $[Ca^{2+}]_{cyt}$ has been firmly established [54] and recent breakthrough studies uncovered the crucial role of glutamate receptor-related channels (GLRs) to proper pollen tube growth and fertility [32]. In addition, channels that account for oscillatory Ca^{2+} influx during tube elongation in Nicotiana, Petunia and Pyrus were shown to be sensitive to gadolinium and lanthanum, that inhibit several types of Ca^{2+} channels [55,56], but not to verapamil and nifedipine, known inhibitors of animal L-type Ca^{2+} channels, which establish the Ca^{2+} gradient before germination starts [33,41]. Moreover, application of cytoskeleton inhibitors to growing pollen tubes revealed the importance of a functional cytoskeleton for regulation of growth pulses [55]. F-actin and also exocytosis oscillate along with the growth rate, whereas $[Ca^{2+}]_{cyt}$ oscillations lag behind them [5,57,58]. These correlations could be combined in a circular model in which increased growth rates lead to influx of extracellular Ca^{2+} through stretch-activated channels (Fig. 2). The consequently elevated Ca^{2+} concentration in turn causes F-actin de-polymerization through actin binding proteins (ABPs like Villin/Gelsolin/Profilin), which slows down the growth rate. Stretch activated Ca^{2+} channels are assumed to be closed (or at least less conductive) under this condition; hence, the cytosolic Ca^{2+} concentration descends which results in reorganization of the actin cytoskeleton and an increasing growth rate [42]. $[Ca^{2+}]_{cyt}$ itself may also trigger its own release from internal stores by activating phosphoinositide-specific phospholipase C (PLC). PLC would release inositol (1,4,5)-trisphosphate (IP_3) which in turn could induce Ca^{2+} release [59]. However, the lack of canonical IP_3 receptors in higher plants questions the assignability of mechanistic principles of IP_3-induced Ca^{2+} release from animal systems to the situation in plant cells [60–62]. Increase in cytosolic free Ca^{2+} also promotes vesicle fusion at the plasma membrane (and thereby exocytosis) [63]. However, it is currently not clear whether this is a direct effect of $[Ca^{2+}]_{cyt}$ or rather indirectly mediated via annexins and synaptotagmins [29,64] since theoretically apical $[Ca^{2+}]_{cyt}$ constantly exceeds the required concentrations to promote exocytosis [36]. This targeted exocytosis serves deposition of new cell wall material required for polar growth. Pollen tubes are not the only plant cell type for which oscillations of $[Ca^{2+}]_{cyt}$ have been reported. However, compared to the Ca^{2+} gradient Ca^{2+} oscillations are not compulsive for the pollen tube growth as for example no regular oscillations could be observed in short pollen tubes of *L. longiflorum* [4,55,65]. Neither in *N. tabacum* nor in Arabidopsis regular oscillations appear to be necessary for pollen tube growth and it has been observed that regular Ca^{2+} oscillations mainly occur in in vitro growing tubes, but rarely in vivo or semi-in vivo [34]. Although this might imply that these in vitro observed oscillations are just epiphenomenal [12], it should be considered that not all of these studies have been performed at the nowadays possible highest resolution in imaging. In order to increase the in vivo resolution two-photon microscopy could be the first choice [66,67]. However, in most studies Ca^{2+} oscillations correlate with oscillations of growth-speed and therefore it appears reasonable to conclude that they coordinate spatial and temporal relationships and represent a self-organizing process which can react homeostatically to perturbations and therefore provide certain stability [55]. Targeted perturbations by for example channel blockers can be used for further analyzing correlations between different oscillating parameters.

4. Calcium mediated regulation of cytoskeleton and vesicle trafficking

Within the tip region of growing pollen tubes a characteristic pattern can be observed, the so called "clear zone" which encompasses the apical 15–20 μm [39,68]. This region is characterized by a cone shaped accumulation of mainly secretory vesicles. Larger organelles (vacuole, nucleus, amyloplasts) are excluded from this region, resulting in the naming clear zone. Mitochondria, peroxisomes and other equally sized organelles are specifically excluded from the most extreme apex, thereby forming an apical submembranar domain of small secretory vesicles in a shape of a tip-inverted cone [69]. In terms of vesicle flow within this region the currently best-supported model indicates that exocytosis occurs at the extreme tip while endocytosis takes place at the shoulders of the tip [2]. Simultaneously, the cytosol exhibits a pattern of cytoplasmic streaming from base to tip with a reverse fountain-like streaming behind the tip [68,70]. A tight regulation of endomembrane trafficking in pollen in general as well as regulation of actin-dynamics are essential for maintaining polarity of this tip-growing cell and both processes are closely linked and both involve Ca^{2+} as a second messenger. While oscillations in $[Ca^{2+}]_{cyt}$ follow growth rate pulses, oscillations of pollen tube growth rates coincide with changes in vesicle dynamics [68]. At growth peaks, massive exocytosis is observed at the very tip of the tube. This is required for deposition of new cell wall material at the apex, where the tube elongates, and for secretion of cell wall modifying proteins. Polarized exocytosis is regulated by signaling pathways involving ROP1, a small GTPase that modulates F-actin dynamics. Similarly as the growth rate, ROP1 activity and F-actin oscillate with the same frequency but ahead of tip localized $[Ca^{2+}]_{cyt}$ [3,42,57,58,71]. ROP1 activity is required for pollen tube tip growth and it is suggested that the distribution of active ROP in the pollen tube apex defines the tip growth region. This is achieved by a self-regulation mechanism integrating positive and negative feedback loops that involve two counteracting

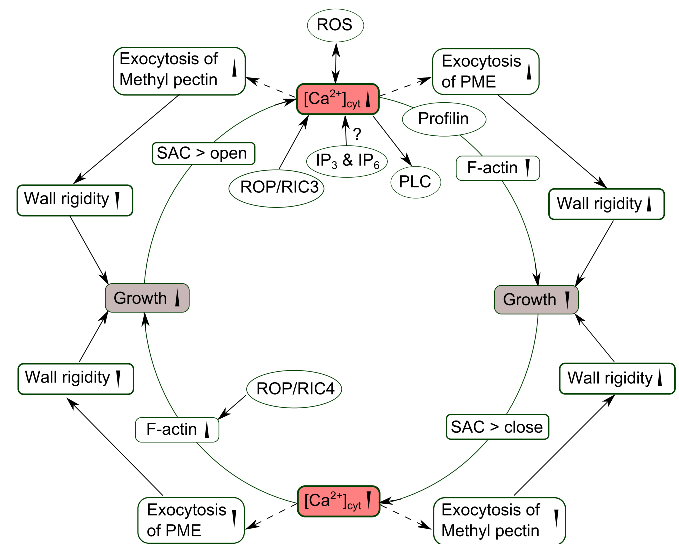

Fig. 2. Schematic model of a self-regulatory network modulating oscillatory growth cycles of an elongating pollen tube. This model integrates changes in concentration of free Ca^{2+} in the cytosol ($[Ca^{2+}]_{cyt}$), apical exocytosis of cell wall material (methyl-pectin) and pectin methyl esterase (PME), changes in cell wall properties, regulation of stretch-activated Ca^{2+} channels (SAC), contribution of F-actin, ROP1 signaling, phospholipase C (PLC) activity, inositol-polyphosphates (IP_3 and IP_6) and reactive oxygen species (ROS). Upright triangles represent increases/up-regulation; inverted triangles represent decreases/down-regulation.

pathways of ROP1 mediated regulation of tip growth (Fig. 2) (reviewed in [2]). The accurate coordination of these two pathways is a prerequisite for proper pollen tube growth. In one pathway interaction of ROP1 with its downstream effector RIC4 provokes F-actin assembly which is necessary for vesicle targeting; however, it blocks vesicle fusion. On the other hand interaction of ROP1 with another downstream effector RIC3 results in Ca^{2+} influx across the plasma membrane [71–73]. This increase in $[Ca^{2+}]_{cyt}$ in the extreme tip causes F-actin disassembly. This process involves the function of actin binding proteins (ABPs) that can either stabilize or depolymerize F-actin. This stabilization/destabilization switch appears to be Ca^{2+} controlled. In lily pollen the Ca^{2+} sensitive ABP LlLIM1 binds F-actin bundles and protects them against depolymerization under low $[Ca^{2+}]_{cyt}$. In high $[Ca^{2+}]_{cyt}$ this protective effect disappears [25]. Conversely, members of the villin/gelsolin family sever actin filaments upon increasing Ca^{2+} concentration [26]. An additional Ca^{2+} sensitive ABP involved in regulation of actin dynamics is profilin. After actin disassembly profilin binds monomeric actin and enhances polymerization [24]. However, the activity of profilin remains diminished as long as $[Ca^{2+}]_{cyt}$ remains high. Remarkably, all these ABPs exhibit an even distribution throughout the tube. However, since they are sensitive to different $[Ca^{2+}]_{cyt}$ a distinct pattern of actin dynamics can be observed (long actin cables extending within the shank and a fringe-like subapical structure formed by shorter, depolymerized actin filaments), as long as the Ca^{2+} gradient is maintained [24,74]. Incidentally, not only actin dynamics is affected by Ca^{2+}. Ca^{2+} has also been reported to influence organelle motility along actin filaments by inactivation of myosin motor activity through binding to its calmodulin light chain, thereby preventing entry of larger organelles into the apical region of the pollen tube [75]. Both the apical increased $[Ca^{2+}]_{cyt}$ and the F-actin disassembly promote exocytosis probably by boosting vesicle fusion [71,76]. The faithfully concerted regulation of all these cell biological processes by Ca^{2+} finally results in the remarkable stability of the sub-cellular organization of the pollen tube despite its highly dynamic growth.

In addition, other important regulation mechanisms as for example phosphoinositide (PI) signaling impact on membrane trafficking and cytoskeletal organization in the growing pollen tubes. PIs are important for regulation of cytoskeletal dynamics, ion channel activity and vesicle trafficking. For example they can control directional membrane trafficking and thereby impact on delivery of cell wall material. The contribution of PI signaling to polar tip growth has been recently comprehensively reviewed by Ischebeck et al. [77].

5. Calcium entry from external sources contributes to pollen tube guidance

As mentioned in Section 3, Ca^{2+} fluxes from the external medium are temporally delayed in comparison to elevation in cytosolic free Ca^{2+}, which points to a function of the cell wall as a storage compartment for this ion. Furthermore, the measured Ca^{2+} fluxes exceed the calculated influx of external Ca^{2+} that would be required to establish the tip-focused Ca^{2+} gradient [50]. This observation also supports the assumption that the pollen tube cell wall stores exceeding Ca^{2+} ions. Indeed the cell wall, which can span up to 0.5 μm, provides immense binding capacities for this divalent cation. At the extreme apex of a growing pollen tube, new cell wall material (mainly methyl-pectin) is secreted [78]. This secretion of material as such initially loosens the apical cell wall matrix, in combination with the existing turgor pressure. Methyl-pectin forms rather loose ionic bonds with Ca^{2+} resulting in a gel with low shear strength [79]. Upon pectin methyl esterase (PME) secretion, methyl-pectin becomes de-methoxylated and consequently cross-linked with free Ca^{2+}. This cross-linking increases cell wall rigidity (Fig. 2). Pollen tubes require an external Ca^{2+} concentration between 10 μM and 10 mM in order to keep the cell wall rigid enough not to burst, but still flexible enough to not stop growing [29].

However, external Ca^{2+} is not only required for cross-linking cell wall components. Germinating pollen grains and elongating pollen tubes also require a continuous external supply of Ca^{2+} in order to establish and maintain the required internal Ca^{2+} gradient. Abundance of external Ca^{2+} even enhances the growing pollen tube's responsiveness to tropic stimuli [80]. Consequently, significant amounts of extracellular free Ca^{2+} have to be present in the stigma and style of the flower. This question has been addressed by Ge et al. who found that in tobacco many Ca^{2+} containing vesicles are present in the exudate at the stigma. Less Ca^{2+} was detected in the transmitting tissue, but 22 h after pollination, the abundance of Ca^{2+} precipitates increased [81].

Considering these diverse functions of Ca^{2+} in such multitude of cell biological processes and the importance of defined Ca^{2+} concentrations as regulatory switches it becomes evident that this ion represents a central hub in the regulatory and signaling networks of the pollen tube.

6. $[Ca^{2+}]_{cyt}$ is fine-tuned by fluxes across the plasma membrane and internal membranes

The fact that the highest Ca^{2+} concentration within the cytosol of the growing pollen tube is locally confined to regions close to the plasma membrane at the extreme apex where most organelles are excluded designates the apoplast as the main Ca^{2+} source for polar Ca^{2+} accumulation. Consequently, Ca^{2+} has to pass the plasma membrane in order to get into the cytosol. One important group of channels that enable Ca^{2+} to enter the cytosol is represented by the previously mentioned stretch-activated Ca^{2+} channels (SACs). SAC channel activities were found to be present in the plasma membrane of protoplasts derived from lily pollen grains and tubes. These channel activities are located at the extreme apex of the tube and at the aperture of the pollen grain, where the tube is supposed to protrude. SACs open in response to deformation of the plasma membrane caused by growth and they are characterized by a high selectivity for Ca^{2+} [53]. It appears conceivable that transmission of tension is brought about via cytoskeletal elements, more precisely by F-actin. In Arabidopsis two genes encoding for potential SACs, namely MCA1 and MCA2, have been identified [82]. Functional characterization of MCA1 in mutant and overexpression lines provided evidence that this protein promotes Ca^{2+} influx in plants upon mechanical stimulation and is critical for plant root growth. However, if MCA1 or related proteins function in pollen tubes has remained unknown [82].

The first identified and molecularly characterized channel for which a contribution to Ca^{2+} influx across the pollen tube's plasma membrane was established is the cyclic nucleotide-gated channel CNGC18 [54]. CNGCs are cation channels that have been shown to have the ability to conduct Ca^{2+} currents (Fig. 1) [62,83]. Overexpression of this plasma membrane localized channel resulted in abnormal pollen tube growth while loss-of-function mutant lines exhibited male sterility. The Arabidopsis genome contains genes for 20 different CNGC isoforms [84,85]. In addition to CNGC18 there are 5 more CNGCs (namely CNGC7, 8, 9, 10 and 16) that are potentially relevant for pollen ion fluxes because their expression is enhanced in pollen grains and/or growing pollen tubes [86–88]. Plant CNGCs appear to become activated by cAMP binding at the cytosolic situated C-terminus and potentially also by hyperpolarization [36,89]. Negative regulation of CNGCs is brought about by calmodulin (CaM)-binding upon increases in cellular Ca^{2+} concentration. Since both CaM and cAMP compete for binding at the channels C-terminus the interplay of Ca^{2+} and cAMP concentration in the pollen provides a means for positive and negative regulation of CNGC channels [90].

An important new twist in our understanding of pollen biology was the recent identification of glutamate receptor-like (GLR) channels as crucial components for Ca^{2+} influx into Arabidopsis pollen by J. Feijó and colleagues (Fig. 1) [32]. GLRs, prior to their discovery

in plants, were only known to mediate Ca^{2+} fluxes in mammal rapid synaptic transmission [91]. They comprise 20 genes in Arabidopsis, at least 6 of which are expressed in pollen [86]. In contrast to mammal glutamate receptors, plant GLRs respond more effectively to D-serine and some other amino acids instead of glutamate [92]. Consistent with this finding Ca^{2+} conductance has been electrophysiologically detected in Arabidopsis and Tobacco pollen tubes in response to D-serine. This conductance was reduced in *Atglr1.2-1* and *Atglr3.7-1* mutants, which exhibit pollen tube growth phenotypes, as well as by treatment with the GLR specific antagonists DNQX and CNQX [32]. Altogether, this very elegant work established by pharmacology and loss-of-function approaches in Arabidopsis and tobacco that GLRs facilitate Ca^{2+} influx across the plasma membrane, thereby modulating the apical $[Ca^{2+}]_{cyt}$ gradient and consequently govern pollen tube growth. These results nicely linked Ca^{2+} to GLR function but due to the applied techniques could not provide final proof of a direct and specific Ca^{2+} channel activity for GLRs. Importantly, this direct evidence for Ca^{2+} permeability of a GLR has recently been reported by Spalding and colleagues who observed in a complementary study that expression of GLR3.4 in HEK cells specifically conferred amino acid gated Ca^{2+} influxes [93]. While animal glutamate receptors can be expressed and electrophysiologically characterized in Xenopus oocytes similar approaches with Arabidopsis GLRs failed to detect amino acid gated activity [94]. This situation is reminiscent of the behavior of other plant ion channels like the slow vacuolar anion channel (SLAC1) which regulates guard cell aperture [95–97] or the Arabidopsis K^+ transporter 1 (AKT1) that regulates root K^+ uptake [98,99]. Both proteins were reported to be inactive when expressed alone in Xenopus oocytes [95,96,98]. Such inactivity may reflect the absence of a plant specific activation mechanism in this heterologous expression system. Quite remarkably, recent studies indeed identified Ca^{2+}-dependent activation mechanisms for SLAC1 and AKT1 [95,98,100,101]. In the case of SLAC1 co-expression of plant kinases among them several CDPKs can bring about phosphorylation and thereby activation of this channel. In the case of AKT1 activation was observed by co-expression of the calcium sensors CBL1 or CBL9 together with their interacting kinase CIPK23. Considering these similarities it is most tempting to hypothesize that plant GLRs are also subject to regulation by CDPK and/or CBL/CIPKs and that investigation of these potential regulatory modules in Xenopus oocytes may significantly advance our understanding of plant GLR regulation.

Another family of proteins that needs to be considered for conveying fluxes of external Ca^{2+} into the cytosol of the pollen tube is represented by the annexins. Annexins form a family of 8 proteins in Arabidopsis and transcriptomic studies of pollen and growing pollen tubes identified at least 2 annexins as being significantly expressed in these cells [86,102]. Among a multitude of other functions, annexins can integrate into the plasma membrane and currently available evidence supports the notion that they may form Ca^{2+} conductive channels. In 2009 Davies and colleagues reported that *Zea mays* annexin preparation caused increases in $[Ca^{2+}]_{cyt}$ when added to protoplasts of *Arabidopsis thaliana* roots expressing aequorin [103]. Most recently, the Arabidopsis loss-of-function mutant for annexin1 (*Atann1*) was found to lack the root hair and epidermal $OH^{\bullet}$-activated Ca^{2+}- and K^+-permeable conductance [104]. Furthermore annexins can forward their own secretion and bind to the actin cytoskeleton [29]. In addition annexins are considered to be involved in prevention of self-fertilization [23] and integration of ROS and Ca^{2+} signaling [105], but more research in this area is clearly needed to address potential functions of annexins in pollen and further elucidate their exact contribution to cellular Ca^{2+} fluxes.

Apparently, most characterized channels responsible for Ca^{2+} influx into the pollen cytosol reside in the plasma membrane, which fits with the fact that the $[Ca^{2+}]_{cyt}$ is the highest in apical regions, close to the plasma membrane. In contrast, little is known about the contribution of internal Ca^{2+} stores to increases in $[Ca^{2+}]_{cyt}$. TPC1, a voltage-gated vacuolar cation channel has recently been put forward by Konrad et al. as a potential candidate, but so far no evidence for its expression/function in pollen has been presented [35]. Compartments like the vacuole and the ER are rather assumed to shape the Ca^{2+} gradient by sequestration of the ion [16,106]. Ca^{2+} pumps that could account for shaping and fine-tuning of the Ca^{2+} gradient in growing pollen tubes are the ER localized type II Ca^{2+}-ATPases ACA2 and ECA1 (Fig. 1) [107,108]. ACA2, an autoinhibited IIB-type Ca^{2+}-ATPase, is expressed in pollen grains [109] and ECA1, a IIA-type Ca^{2+}-ATPase, is inhibited by cyclopiazonic acid (CPA), a specific inhibitor for plant P-type IIA Ca^{2+}-ATPases [110], which affects the growth rate and $[Ca^{2+}]_{cyt}$ dynamics of pollen tubes [34]. These low-capacity, high-affinity pumps hydrolyze ATP while moving Ca^{2+} against its concentration gradient into the ER where it is bound by calreticulin [111–114]. Ca^{2+} concentrations inside the ER range from 100 to 500 μM [36]. Interestingly, these Ca^{2+} pumps are also a target of Ca^{2+}/CaM, which activates them in order to decrease $[Ca^{2+}]_{cyt}$ and to prevent cell death [112]. ACA2 moreover can be phospho-inhibited by CPK1, as long as no CaM is bound [108]. One more relevant Ca^{2+} pump among the 14 ACAs in Arabidopsis is ACA9. ACA9 is evenly distributed in the plasma membrane of growing pollen tubes and it is assumed to sequester cytosolic Ca^{2+} into the apoplast. T-DNA insertion mutants revealed partial male sterility [40]. As well as ACA2, the autoinhibited ACA9 is a target of Ca^{2+}/CaM activation, a fact that suggests that the majority of activated ACA9 is present in the tip region, due to the tip-focused Ca^{2+} gradient. In terms of Ca^{2+} sequestration into the vacuole of pollen tubes cation Ca^{2+} exchangers (CAX) represent potential candidates for fulfilling such a function in pollen. Six of the 14 members comprising family in Arabidopsis are expressed in pollen (namely CAX 3, 4, 8, 9, 11 and MHX1), with CAX 4 and CAX 9 being specifically expressed in pollen [86,88]. However, so far no function in pollen germination and tube growth has been established for CAX Ca^{2+} exchangers.

7. Sensing Ca^{2+}

In addition to the essential regulatory function of defined concentrations of Ca^{2+} for polar pollen tube growth by the modulation of for example vesicle transport or actin dynamics, Ca^{2+} signals are perceived by Ca^{2+} sensor proteins that decode the information presented in specific Ca^{2+} signatures into distinct downstream signaling responses. Over the past decade the importance of this second level of calcium signaling for plant response reactions and development has been increasingly appreciated [23,115,116]. These sensors are Ca^{2+} binding proteins that specifically modulate signaling cascades resulting in a multitude of defined responses as for example activation or inactivation of ion channels or transporters. This tight regulation of ion fluxes is required in order to establish and maintain ion gradients across membranes within the cell and with respect to the apoplast. In order to perceive changes in $[Ca^{2+}]_{cyt}$ and transduce them as a specific signal, sensors have to bind Ca^{2+}. Ca^{2+} binding is brought about by a helix-loop-helix motif, the so called EF-hand motif. Arabidopsis possesses at least 250 different EF-hand containing Ca^{2+} sensors with varying Ca^{2+} affinities as well as diverse subcellular localization patterns and different target specificities [117]. All these features make these Ca^{2+} sensors a multifaceted decoding system for a diversity of Ca^{2+} signals [115,117]. Plants are equipped with a complex array of Ca^{2+} decoding proteins encompassing at least 4 different families of EF-hand Ca^{2+} sensors which can be divided into two groups. One group is represented by "sensor relays" which do not possess any enzymatic activity themselves but transduce Ca^{2+} signals through conformational change determined protein-protein interactions upon Ca^{2+} binding. The second group has been designated as "sensor responders" since they directly transduce Ca^{2+} signals via phosphorylation of target proteins [118]. The

families of calmodulins (CaM) and calmodulin-like (CML) proteins belong to the group of “sensor relays” which transduce the Ca^{2+} signal via Ca^{2+}-dependent protein-protein interactions. Another family of Ca^{2+} sensors, the calcineurin B-like (CBL) proteins, also belongs to the group of “sensor relays” since they lack an enzymatic activity. However, since CBL proteins specifically form complexes with protein kinases designated as CBL-interacting protein kinases (CIPKs) they could also be referred to as a form of bimolecular “sensor responders” [117]. Bona fide “sensor responders” are represented by the family of Ca^{2+}-dependent protein kinases (CDPKs) that contain an EF-hand domain as well as a kinase domain. In Arabidopsis there are 7 CaMs, 50 CMLs, 10 CBLs, 26 CIPKs and 34 CDPKs annotated in the genome. Recent analyses of microarray data suggested that several members of these families are strongly or specifically expressed during pollen germination and tube growth. This refers to at least one isoform of the CaMs, 18 CMLs (9 of them pollen-specifically), 17 CDPKs (5 of them pollen-specifically) and at least 4 CBLs are expressed in pollen and are therefore likely involved in Ca^{2+} signal transduction during germination and tube growth [86,119]. Accordingly, several genetic studies have revealed functions of calcium binding proteins in pollen tubes. For the CaM isoform 2/3/5 a loss-of-function allele of the Calmodulin2 gene has been reported to affect pollen germination in Arabidopsis [120]. CaM has shown to be present throughout the cytosol of the tube [121] but since its activity depends on Ca^{2+}, a corresponding tip-focused gradient of active CaM could be observed [122]. Thereby active CaM located in the tip region can negatively regulate targets like CNGCs, activate targets like ACAs and promote secretion. Considerably less information is available about CML protein function in pollen. Although several CMLs appear to be preferentially expressed in pollen, no function could be assigned to these proteins so far. However, transient overexpression of CML21::GFP in tobacco pollen tubes caused reduced elongation and slightly increased width of the tip [119]. A comparable phenotype has been observed for CBL3 overexpression in the same study. However, no further insights into the function of this calcium sensor in the growing pollen tube were provided. The role of CIPKs in pollen tubes is currently completely unknown. In contrast to CIPKs there is already some evidence for a function of CDPKs in pollen. Overexpression studies of *Petunia inflata* CPK1 and CPK2 and Arabidopsis CPK24, CPK32 and CPK34 as well as mutant analyses of Arabidopsis CPK17 and CPK34 revealed that these proteins regulate polar growth of the pollen tube [106,119,123]. Considering that members of both kinase families have recently been shown to regulate a wide range of transporters and channels for ions like K^+, Cl^- and NO_3^- [96,100,124] it appears very likely that the pollen tube expressed CIPKs and CDPKs fulfill similar functions in this model system. Therefore it appears save to predict that detailed studies of the function of these kinase proteins and their potential targets in pollen tubes will provide important insights into regulatory mechanisms governing pollen tube biology. Moreover, as mentioned above, these calcium-regulated kinases represent prime candidates for mediating the regulation of GLRs and CNGCs. Considering the steep gradient of cytoplasmic Ca^{2+} in pollen tubes such regulatory modules would provide an elegant and intuitive mechanistic basis of a Ca^{2+}-dependent regulation of Ca^{2+} fluxes into the pollen apex. Such Ca^{2+}-dependent phosphorylation of ion transporters and channels may only present one facet of the general importance of post-translational protein modification by phosphorylation in pollen and pollen tubes. Since the mature, dehydrated pollen grain already contains all necessary transcripts and proteins required for germination and initial pollen tube growth [125], post-translational regulation of protein activity is likely to present the prime mechanism for fast and extensive changes in ion fluxes, vesicle transport and cytoskeleton organization that accompany pollen development. In this regard, a recent phospho-proteome study of the soluble phospho-proteome of mature Arabidopsis pollen identified almost 600 phosphorylated proteins [125]. Interestingly, two members of the CDPK family CPK11 (AT1G35670) and CPK4 (AT4G09570) were identified as being phosphorylated in pollen in this study. Our own inspection of this data set revealed at least 5 not further specified EF-hand calcium binding proteins as being phosphorylated in pollen. Of these EF-hand proteins 3 were classified as being pollen enriched since they have not been detected in the large-scale phospho-proteome database. Quite remarkable two of these non-specified EF-hand proteins represent CMLs (AtCML25, AT1G24620.1 and AtCML26, AT1G73630.1). These findings underscore the potential function of calcium sensors in pollen and more specifically identify CMLs as proteins that may deserve closer functional investigation. Moreover, these data point to a potential more general occurrence of phosphorylation of EF-hand proteins as their regulatory mechanism, which has established for Arabidopsis CBL proteins [126]. From all this accumulating evidence it is becoming quite evident that there is an urgent need to advance our understanding of how Ca^{2+} signals are decoded in pollen and mechanistically lead to adequate responses in order to enable fertilization.

8. Crosstalk with other signaling networks

Certainly, Ca^{2+} signaling does not occur in an insulate network without signal integration and coordination with other signaling systems. There are many points where communication with other signaling networks has to occur and actually occurs (Fig. 2). In addition to examples that have already been provided in previous sections of this review we here will emphasize some interconnections and convergence points of Ca^{2+} signaling with other signaling pathways. PI signaling is beyond doubt an important instance controlling directional membrane trafficking in pollen tubes and thereby influencing polar growth processes. There are several points known where PI and Ca^{2+} signaling appear to converge as for example the aforementioned PI-specific member of the PLC family. Plant PLCs belong to the PLCζ class, which harbor EF-hands that bring about Ca^{2+} regulation of these proteins [61]. Also the activity of inositol polyphosphate kinase (IPK2α) which converts IP_3 into inositol-(1,3,4,5,6)-pentakisphosphate (IP_5) exerts influence on the abundance of IP_3 as well as on levels of IP_3 derivatives such as IP_5 and IP_6. Reduction of IPK2α transcript levels in Arabidopsis leads to enhanced pollen germination and tube growth which is probably caused by inositol polyphosphate mediated regulation of Ca^{2+} signaling [127]. But there is not only this correlation between inositol polyphosphate metabolism and Ca^{2+} signaling. PI controlled membrane trafficking is likely also interconnected with Ca^{2+} signaling as for example type β PI4-kinases are assumed to be recruited to trans-Golgi vesicles by Ca^{2+} sensing proteins such as frequenin and CBLs [77]. Another link between these two networks is provided by OsPBP1, a single C2-domain containing protein which binds phospholipids Ca^{2+}-dependently [128]. Interestingly, Ca^{2+} binding to OsPBP1 entails its translocation from the cytoplasm and nucleus to the plasma membrane. OsPBP1 is preferentially expressed in the pistil and pollen but down-regulated during pollination. The function of OsPBP1 is so far unknown, but it represents a connection between Ca^{2+} and PI signaling that is required for pollen fertility as demonstrated by analyses of antisense OsPBP1 transgenic lines [128]. Interlaced with phospholipid and Ca^{2+} signaling is the signaling of ROS generated by Rboh (respiratory burst oxidase homolog) NADPH oxidases which are located in lipid microdomains in the apical plasma membrane of growing pollen tubes [129]. The requirement of Rboh generated ROS for pollen tube growth and for polarized plant cell growth in general has been established by pollen germination experiments combined with Rboh-specific antisense oligodeoxynucleotide mediated down-regulation of Rboh expression, by ROS scavengers and by an Rboh inhibitor, respectively [130]. Exogenous application of H_2O_2 was able to rescue the impaired tube growth. A direct connection to Ca^{2+} signaling is provided by the fact that binding of Ca^{2+} activates

Rbohs [131]. This activation mechanism probably occurs synergistically with phosphorylation of Rbohs by SnRK2-type kinases [132,133] and unknown Ca^{2+}-activated kinases [134]. Moreover, binding of acidic phospholipids and action of Rac/Rop GTPases also can enhance Rboh activity [131]. Activation of an Rboh (RHD2) through Ca^{2+} binding has previously also been shown in Arabidopsis root hairs [134]. Rboh activity leads to H_2O_2 mediated activation of currently unknown Ca^{2+} channels which in turn causes an increase in the cytosolic Ca^{2+} level (Figs. 1 and 2). Recently, these observations have been extended in terms of pollen tube initiation of lily and kiwifruit pollen, as well as of tobacco and olive pollen where locally restricted ROS production at an aperture of the pollen grain was required for outgrowth at that particular aperture [131,135,136]. Comparable to ROS nitric oxide (NO) also appears to modulate intracellular Ca^{2+} levels through regulation of Ca^{2+} channels in the plasma membrane as well as in internal membranes and in fact, both types of signaling molecules are acting in concert [137,138]. Adjustments of ROS and NO levels have been described to be implicated in pollen-stigma recognition and targeting of pollen tubes to the ovules [139,140]. As distinguished from ROS, NO can activate guanylyl cyclase and thereby possibly indirectly activate CNGCs through increases in cGMP levels [85,138]. Increases in cytosolic Ca^{2+} again can promote negative regulation of CNGCs mediated by CaM and thereby terminate the influx of Ca^{2+}.

Recent findings suggest that internal Ca^{2+} levels can even regulate (at least to some extend) outward rectifying channels for Cl^- and NO_3^- which are assumed to electrostatically counterbalance fluxes of cations [48]. This finding directly links the internal Ca^{2+} to the regulation of levels of important anions involved in pollen tube growth. However, it is so far not clear how Ca^{2+} exerts its influence on these channels. Despite these listed interconnections of Ca^{2+} with other signaling systems there is likely a vast amount of interactions and signaling coordination that still awaits discovery and elucidation. Advancing this research field of pollen biology will be especially crucial to gain an integrated understanding of this model system.

9. Conclusions and perspectives

During pollen germination and polar growth of pollen tubes many ions are involved and function in concert in diverse regulatory processes required to enable a single growing cell to span an incredible distance without any cell division. Central among these ions and interconnecting the modulation of many cellular processes is Ca^{2+}. Well-directed adjustments of $[Ca^{2+}]_{cyt}$ which are crucial for full fertility of a pollen require channels that allow for controlled Ca^{2+} fluxes across membranes (Fig. 2). SACs as one of the first characterized Ca^{2+} selective channels most likely account for apical Ca^{2+} influxes in growing pollen tubes [53]. The further advancement of our understanding of the generation of the polar Ca^{2+} gradient in pollen will require the determination of the molecular identity of the SACs. Recent findings also point to cation channels such as CNGCs and especially the lately identified GLRs as being responsible for Ca^{2+} influx [32,54,93]. However, many aspects of Ca^{2+} channel regulation and especially Ca^{2+} signal decoding and transduction are still poorly understood in pollen germination and tube growth. Future research is needed to address a diversity of questions in order to stepwise elucidate the operating mode of the extremely complex Ca^{2+} signaling network in this model system. During the past years great advancements have been made in understanding Ca^{2+} decoding processes in plants in general. Plants feature an extensive set of Ca^{2+} binding proteins that have the ability to sense complex Ca^{2+} signals and translate them into regulatory events at different levels, such as transcription or post-translational modification (e.g. phosphorylation). The recent progress that has been achieved in Ca^{2+} signaling research of other model systems like guard cells and of processes like abiotic stress responses should provide a most useful conceptual framework for a rapid advancement of our understanding of Ca^{2+} in pollen biology. In this regard, it will be most interesting to further explore on the one hand how Ca^{2+} signals are generated in pollen and on the other hand, how these signals are integrated by Ca^{2+} signaling networks, as well as the interrelationship with other complex signaling networks such as ROS, cyclic nucleotide and phospholipid signaling.

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