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The ins and outs of Ca^{2+} in plant endomembrane trafficking

Ellie Himschoot[1,2,4], Roman Pleskot[1,2,3,4], Daniël Van Damme[1,2] and Steffen Vanneste[1,2]

Trafficking of proteins and lipids within the plant endomembrane system is essential to support cellular functions and is subject to rigorous regulation. Despite this seemingly strict regulation, endomembrane trafficking needs to be dynamically adjusted to ever-changing internal and environmental stimuli, while maintaining cellular integrity. Although often overlooked, the versatile second messenger Ca^{2+} is intimately connected to several endomembrane-associated processes. Here, we discuss the impact of electrostatic interactions between Ca^{2+} and anionic phospholipids on endomembrane trafficking, and illustrate the direct role of Ca^{2+} sensing proteins in regulating endomembrane trafficking and membrane integrity preservation. Moreover, we discuss how Ca^{2+} can control protein sorting within the plant endomembrane system. We thus highlight Ca^{2+} signaling as a versatile mechanism by which numerous signals are integrated into plant endomembrane trafficking dynamics.

Addresses
[1] Ghent University, Department of Plant Biotechnology and Bioinformatics, 9052 Ghent, Belgium
[2] VIB Center for Plant Systems Biology, 9052 Ghent, Belgium
[3] Institute of Experimental Botany, Czech Academy of Sciences, Rozvojova 263, 16502 Prague, Czech Republic

Corresponding author: Vanneste, Steffen (stnes@psb.vib-ugent.be)
[4] These authors contributed equally.

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Introduction

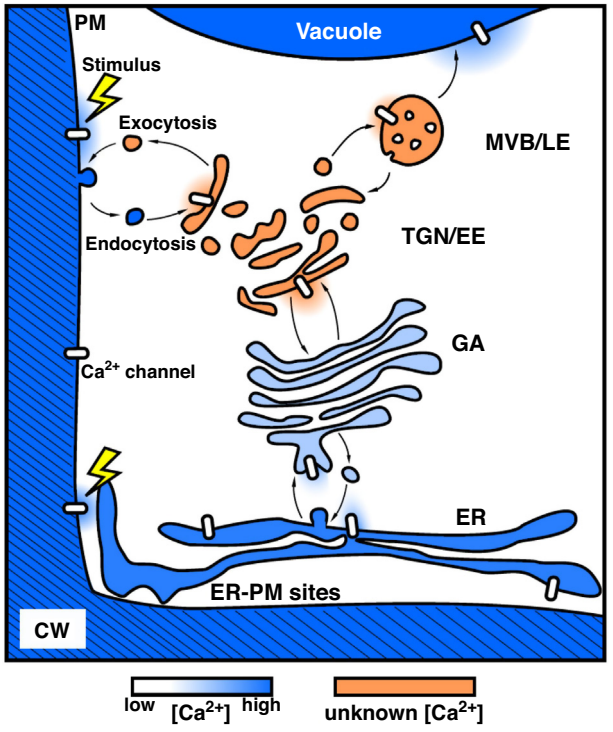
The alkaline earth metal calcium is one of the most abundant elements on earth. Much of the Ca^{2+} as we know it is present as calcium phosphate in bones and teeth, or as calcium carbonate in lime stone, pearls and shells. These prominent forms of Ca^{2+} have been generated by living organisms that employ calcium's chemical propensity to precipitate anions such as phosphates and carbonate as scaffolds and protection for their bodies. Besides such structural functions, Ca^{2+} acts in all living organisms as a second messenger in the context of a wide range of cellular processes and signaling cascades. In plants, the second messenger function of Ca^{2+} is best described in the context of responses to biotic and abiotic stress, symbiosis, tip growth of pollen tubes and root hairs and egg cell fertilization. For a detailed overview of the complexities that underlie Ca^{2+} signal generation and transduction in plants, the reader is referred to a number of outstanding reviews on the topic and references therein [1–4].

Importantly, one of the key aspects of Ca^{2+} signaling, which is often overlooked, is its local nature, acting in microdomains within the cell. To avoid toxic effects of high Ca^{2+}, e.g. at the level of phosphate metabolism, cells keep cytoplasmic Ca^{2+} levels very low (typically in the submicromolar range: 100–200 nM), via energy consuming Ca^{2+} transport into the apoplast and intracellular organelles, which can have Ca^{2+} concentrations in the millimolar range (reviewed in [5], Figure 1). The resulting steep concentration gradients over the cellular membranes thus allow to rapidly generate a strong local cytoplasmic Ca^{2+} increase by opening a few Ca^{2+} channels. Moreover, electrostatic interaction with anionic moieties buffer the cytoplasm against rapid Ca^{2+} diffusion, resulting in sharply defined microdomains of high Ca^{2+} (>100 μM) in the direct proximity (+/−100 nm) of activated Ca^{2+} channels [6–8]. Therefore, such discrete Ca^{2+} signals allow for regulation of subcellular processes with sub-micrometer precision.

In animal cells, local Ca^{2+} signaling is well known for its role as a regulator of endomembrane trafficking; the process in which proteins are selectively moved between interconnected subcellular endomembrane compartments via tightly regulated membrane budding, transport and fusion. This paradigm is nicely illustrated in neurotransmission, where local cytoplasmic Ca^{2+} signals in axon termini trigger the exocytosis of neurotransmitter-filled vesicles to activate downstream neurons [9]. Therefore, it is easy to envision that Ca^{2+} will also play a major role in plant endomembrane trafficking. However, despite the common evolutionary origin of Ca^{2+} signaling in Eukaryotes, it is often difficult to identify conserved molecular mechanisms of Ca^{2+} signaling between animals and plants, as the large evolutionary distance also allowed for a remarkable Kingdom-specific diversification and specializations within the Ca^{2+} toolset [2,10•,11].

In this review, we provide an overview of how Ca^{2+} regulates plant endomembrane trafficking and discuss possible underlying molecular mechanisms. In particular, we will discuss the interplay between Ca^{2+} and phospholipids, the direct effect of Ca^{2+} on the endomembrane machinery and how luminal Ca^{2+} levels are connected to protein sorting in the endomembrane system. Despite the well described functions of Ca^{2+} in plant stress responses, limited information is available regarding the cell biological, local relevance of Ca^{2+} signaling in plants. Therefore, we will at some points resort to reasonable extrapolations from observations in metazoans to bridge some gaps in our knowledge in plants.

Figure 1



Calcium distribution within the plant endomembrane system. In blue: summary of experimental estimates of Ca^{2+} levels, being high (millimolar range) in the apoplast, the endoplasmic reticulum and vacuole, intermediately high (700 nM) in the Golgi Apparatus (GA) and low in the cytoplasm (submicromolar range). In orange: Unknown Ca^{2+} levels across the endomembrane system. Extrapolations from the animal field and biochemical observations suggest the following: (1) Vesicle budding does not allow exclusion of Ca^{2+} from the lumen of vesicles during budding from its donor organelle and is illustrated as the budding vesicle having the same Ca^{2+} concentration as its donor organelle. (2) Ca^{2+} can be released from the vesicles during trafficking or is deposited to the target organelle upon fusion. (3) Each endomembrane compartment is postulated to be decorated with Ca^{2+} channels that can generate Ca^{2+} signals for regulation of e.g. membrane fusion events. (4) Stress and other stimuli activate Ca^{2+} channels that generate Ca^{2+} signals that are focused around the mouth of the Ca^{2+} channel. (5) Because VSRs are dissociated from their ligands in the *trans*-Golgi Network/Early Endosomes (TGN/EE), we postulate that this reflects Ca^{2+} levels being lower in the TGN/EE than in the GA. (6) The presence of the Ca^{2+} ATPase ECA3 in Multi Vesicular Bodies/Late Endosomes (MVB/LE) suggests the Ca^{2+} levels to be at least higher than in the cytoplasm, but are difficult to further resolve relative to those in the TGN/EE.

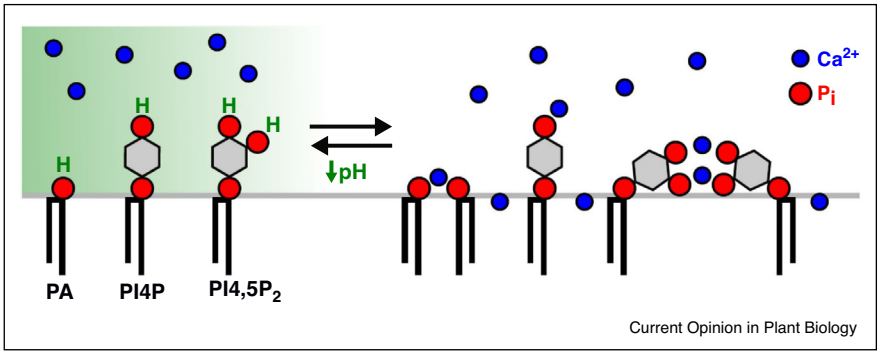
Calcium sensing via anionic phospholipids

Although only representing a minor fraction of the total membrane lipids, anionic phosphoinositides and phosphatidic acid are key endomembrane components. Each endomembrane compartment is hallmarked by a specific phosphoinositide signature that determines differential protein recruitment [12••,13,14], which underlies important connections to processes such as regulation of cytoskeleton dynamics [15,16], exocytosis [17,18] and endocytosis [19–21]. Recently, a unique electrostatic signature controlled by phosphatidylinositol 4-phosphate (PI4P) was described for the plant plasma membrane and the cell plate of dividing cells [12••]. The membrane surface charge was found to control the membrane recruitment of several peripheral membrane proteins, with the forming cell plate being the most electronegatively charged membrane. The net negative charge of anionic phospholipids depends on local pH and allows for dynamic pH-dependent modulation of protein-membrane interactions (see Box 1). This biochemical principle is probably universally valid as a similar pH sensing function has been described in yeast and animals for phosphatidic acid [22,23] (Figure 2). Consistently with such an electrostatic regulation at the plasma membrane, the dynamics of clathrin-mediated endocytosis were

Box 1 Electrostatic interactions of phospholipids with ions

Non-covalent interactions are key modes of interaction in biological systems; they play essential roles in protein folding, DNA replication or enzyme catalysis. Electrostatic interactions, hydrogen bonds and van der Waals interactions are the three fundamental non-covalent interactions. Electrostatic interactions between two charged molecules or ions could be either repulsive in the case of same charges or attractive in the case of opposite charges. The presence of acidic phospholipids in the cytoplasmic face of the eukaryotic membranes results in a significant net negative surface charge [75], which recruits mobile counterions from the cytoplasm that can cause pronounced changes in membrane curvature and surface patterning [75,76]. Phosphatidic acid, PI4P and PI(4,5)P_2 contain phosphate group(s) as a part of their head group region (Figure 2). Protonation state, i.e. the negative charge (−1 or −2) of the phosphate group, changes within a physiological pH range (i.e. 6.9–7.9) [77]. Changes in the negative charge of the phosphate group affect interaction with positively charged ions and/or proteins and thus enable anionic phospholipid containing the phosphate group to work as pH sensors [22,23]. Moreover, electrostatic interaction allows to concentrate Ca^{2+} in the direct proximity of the membrane, exceeding ten times the bulk concentration in the cytoplasm [78]. On the one hand, Ca^{2+} could act as a charge bridge connecting phospholipids and proteins as was recently shown for mammalian SYNTAXIN1 [79]. On the other hand, specific binding of Ca^{2+} to PI(4,5)P_2 changes the phospholipid head group conformation and inhibits PI(4,5)P_2 recognition by pleckstrin homology (PH) domains [29••].

Figure 2



Schematic representation of the pH-dependent interplay between Ca^{2+} and anionic phospholipids. Ca^{2+} interacts electrostatically with the phosphate head groups in phosphatidic acid (PA) and phosphoinositides, such as phosphatidylinositol 4-phosphate, PI4P and phosphatidylinositol 4,5-bisphosphate, $PI(4,5)P_2$ (right). Interaction of Ca^{2+} neutralizes negative charges of the lipids, can induce clustering and lipid head group tilting, which stimulates or interferes with specific protein-lipid interactions. Under conditions of low pH, Ca^{2+} dissociation from the lipids is stimulated (left), thereby reverting the Ca^{2+} dependent effects on phospholipids and their interaction with proteins.

shown to depend on anionic phospholipids [21], and cytoplasmic pH [21,24•].

The bivalent cation Ca^{2+} binds to deprotonated anionic phospholipids and has, besides effects on protein interactions, many biophysical consequences for membrane organization (reviewed in [25]). Importantly, the positive charge of Ca^{2+} modulates the effective charge of the cytoplasmic leaflet of the endomembranes, and could thus interfere with electrostatic interactions at these positions. This is relevant during membrane fusion, where Ca^{2+} facilitates contact between two proximal membranes by bridging lipid head groups [26]. However, several recent studies have suggested that the interaction of Ca^{2+} with membranes is more complex and is not just limited to Ca^{2+}-regulated vesicle fusion [27••,28,29••].

Besides modulating the electrostatic properties of the membrane, Ca^{2+} binding to the phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) head group and carbonyl regions leads to confined lipid head group tilting and conformational rearrangements that modify lipid recognition specificity [29••]. Together, these findings imply that Ca^{2+} allows to rapidly modulate the local electrostatic environment and presentation of phospholipids in membranes for differential recruitment and/or activity of phospholipid-binding proteins.

Conversely, the Ca^{2+} buffering capacity of membranes can also modify Ca^{2+} signaling, as lipids can display high lateral diffusion rates within the membrane [27••], and can locally release the Ca^{2+} bound to anionic phospholipids upon cytoplasmic acidification, or by enzymatic removal of the phosphate head group. Altogether, this intricate electrostatic interplay between Ca^{2+} and phospholipids highlights a fundamental mechanism by which Ca^{2+} signals are integrated in controlling endomembrane trafficking.

Calcium sensing proteins that regulate endomembrane trafficking

Endomembrane trafficking encompasses highly orchestrated membrane budding and fusion and cytoskeleton-based transport in which Ca^{2+} sensing proteins are involved. Calcium sensing is usually conferred by the presence of one or more highly conserved Ca^{2+} binding domains [10•]. For example, the Arabidopsis genome encodes for at least 250 proteins, including prominent regulators of endomembrane trafficking, with one or more EF-hands, archetypal Ca^{2+} binding sites [30]. Several endocytic regulators as well as subunits of the TPLATE adaptor complex (TPC) were reported to contain EF-hand motifs [31,32]. Although not experimentally validated, Ca^{2+} binding via the EF-hands in the TPC may explain the potent and immediate effect of caffeine treatment on the removal of TPC subunits, as well as clathrin, from the growing cell plate during cytokinesis [33•]. In this model, caffeine-induced Ca^{2+} release from intracellular stores is sensed directly by TPC subunits which, in combination with electrostatic effects at the highly electronegative cell plate membrane, disrupts the membrane recruitment of the TPC. The released Ca^{2+} is not efficiently dissipated during cell plate expansion. Following cell plate attachment to the plasma membrane, membrane recruitment of the TPC and other CME machinery rapidly recovers, suggesting restoration of Ca^{2+} dissipation after establishing a continuum between the cell plate lumen and the apoplast. Next to endomembrane trafficking, many regulators of the actin and microtubule cytoskeleton are known to be regulated by Ca^{2+} [34,35,36•]. In addition to Ca^{2+}-dependent regulation of somatic cell shape and growth, this is highly relevant for

tip growing pollen tubes, where an oscillating tip-focused Ca^{2+} gradient instructs targeted secretion in a positive feedback regulation between Ca^{2+} and ROS production [37,38].

In animals, Ca^{2+} sensing via Calmodulin (CaM) fine-tunes core regulatory mechanisms of vesicle tethering and fusion at the level of RAB GTPase and SNARE (soluble-*N*-ethyl-maleimide sensitive fusion factor attachment protein receptor) activity (Reviewed in [39]). In plants, canonical CaM has not yet been reported to be enriched in RAB or SNARE enriched endosomal fractions [40,41]. However, Arabidopsis CALMODULIN-LIKE4 (AtCML4) and AtCML5 are CaM domain-containin membrane proteins that reside in endosomal populations that overlap with Golgi and MVB/LE markers [42•], suggesting that they could modulate Ca^{2+}-dependent vesicle trafficking at these endosomal compartments.

Two other classes of bona fide Ca^{2+} sensing proteins seem to be involved in endomembrane trafficking: ANNEXINs and SYNAPTOTAGMINs. ANNEXINs are conserved, multifunctional Ca^{2+} binding proteins that are involved in membrane trafficking, membrane-cytoskeleton interactions, and can even generate Ca^{2+} channels. Plant ANNEXINs display many similarities to their metazoan counterparts (reviewed in [43]). As their name suggests, they function to bring together/annex membranes, a process intrinsic to endomembrane trafficking. Consistently, purified maize ANNEXINs (ZmANN33/35) could potentiate Ca^{2+}-regulated exocytosis in root cap protoplasts [44], and overexpression of *Arabidopsis* ANNEXIN5 (ANNAT5) renders pollen tube growth more resistant to the exocytosis-inhibiting fungal toxin, Brefeldin A [45]. In addition, ANNAT4 co-purifies with the Qa-SNAREs SYP121, SYP122, SYP123, SYP21 and SYP22 [40], suggesting that plant ANNEXINs act in conjunction with the core membrane fusion machinery. However, genetic evidence supporting the role of plant ANNEXINs in membrane trafficking remains scarce. One clear example is that protoplasts expressing a RNAi construct directed against ANNAT3 display defective segregation of TGN/EE and MVB/LE markers, connecting ANNAT3 to vacuolar trafficking [46•].

On the other hand, plant SYNAPTOTAGMINs, such as SYT1, act as molecular tethers between the ER and the PM [47,48] . In their C-terminal domains, they have Ca^{2+} sensing C2 domains for Ca^{2+}-dependent interaction with phospholipids [49]. Interestingly, expression of a truncated SYT1 construct in tobacco inhibits endocytosis as indicated by a reduced uptake of the endocytic tracer dye FM4-64 and aberrant localization of the endosomal marker RabF1/Ara6-GFP [50]. Similarly, the structurally related *Xenopus* EXTENDED SYNAPTOTAGMIN2 acts as an early endocytic adaptor for the rapid phase of endocytosis of activated Fibroblast Growth Factor Receptors [51], suggesting a conserved functionality for such Ca^{2+} sensing proteins in regulation of endocytosis in animals and in plants.

Together, these examples illustrate how Ca^{2+} sensing proteins control endomembrane trafficking.

Calcium connects endomembrane trafficking to membrane integrity

One of the most fundamental battles that cells are engaged in, is the continuous fight to preserve their membrane integrity in an ever-changing environmental and developmental context, and this involves a strict coordination with endomembrane trafficking. A striking example is the reversible change in guard cell surface, up to 40%, during stomatal movement, that is dependent on concomitant changes in endocytosis and exocytosis rates due to the limited elasticity of the PM [52•,53]. Similarly, cell volume changes triggered by changes in turgor pressure during osmotic challenges are followed by changes in endomembrane trafficking. When plants are subjected to acute hyper-osmotic stress, endocytosis (including bulk internalization) increases and exocytosis decreases, while hypo-osmotic conditions have the opposite effect [54•,55,56]. The functional connection to membrane integrity systems is illustrated by mutants defective in early endocytic steps being more sensitive to hyperosmotic stress compared to controls [54•]. This is consistent with a model in which stress hijacks endomembrane trafficking processes to lower membrane tension and preserve cellular integrity by changing the balance between endocytosis and exocytosis. Given that Ca^{2+} signals are readily elicited upon stress perception, it is easy to envision a role for Ca^{2+} sensing regulators of endomembrane trafficking in membrane integrity preservation, which is the case for plant ANNEXINs and SYTs. Indeed, mutants *annat1* and *annat4*, but also overexpressors of ANNAT8 are tolerant to abiotic stress [57,58]. Given their presumed conserved role in membrane fusion (see above), plant ANNEXINs could thus be involved in a patch-like membrane repair mechanism in which vesicles are fused as a 'membrane patch' across the PM breach [43]. On the other hand, *syt1* mutants are hypersensitive to mechanical stress [47], hyperosmotic stress [49] and freezing [59], while overexpression of a truncated SYT1 results in inhibition of endocytosis [50]. These examples illustrate the Ca^{2+}-dependent interconnection between controlled endomembrane trafficking and membrane integrity preservation mechanisms.

Calcium and protein sorting

Endomembrane trafficking involves highly regulated sorting of specific cargoes of the secretory and the endocytic pathways for secretion, recycling and/or degradation. Vacuolar Sorting Receptors (VSRs) divert vacuolar cargoes away from the default secretory pathway into the vacuolar pathway, as mutants defective in VSR function

missort vacuolar cargoes to the apoplast [60•,61,62]. For reasons of cellular economy, the VSRs do not follow their ligands into the vacuole, but become recycled to the TGN/EE via retrograde transport from MVB/LE. Therefore, VSRs have to dissociate from their ligands before being released in the intraluminal bodies of the MVB/LE. In mammalian cells, this dissociation is induced by rapid acidification and a loss of Ca^{2+} from the maturing endosome, based on pH-sensitive and Ca^{2+}-sensitive ligand binding [63]. Similarly, in plants, Ca^{2+} strongly stabilizes receptor-ligand interactions, albeit independently of pH [64•,65]. The latter is consistent with the absence of a dramatic acidification within the anterograde endosomal pathway [66]. Interestingly, targeted retention of soluble VSRs in different subcellular compartments demonstrated that the VSR-ligand interaction occurs in the ER and the GA, but not the TGN/EE and MVB/LEs [67••]. This raises the possibility that Ca^{2+} release from the endosomal lumen could promote VSR-ligand dissociation. Consistently with this notion, the GA has a relatively low Ca^{2+} concentration (700 nM) [68] compared to the expected Ca^{2+} levels in the ER [5]. Moreover, in this context, one might expect even lower Ca^{2+} levels in the TGN/EE to effect VSR-ligand dissociation. Therefore, it will be of interest to further dissect the Ca^{2+} dynamics along the endomembrane system and the underlying homeostasis mechanisms (Figure 1). Prime candidates for endosomal Ca^{2+} homeostasis are the Ca^{2+} ATPase ECA3 which resides in MVB/LE [69], and an uncharacterized member of the OSCA-type Ca^{2+} permeable cation channels, whose loss of function results in missorting of vacuolar cargoes to the apoplast [60•].

Conclusions and perspectives

The flexibility of plant growth and development depends largely on its ability to integrate numerous environmental stimuli and endogenous cues. This involves a tight coupling with endomembrane trafficking as illustrated by the modulation of cellular signal transduction and transport capacities in the plasma membrane through endocytic regulation of receptors and transporters [70–74]. Ca^{2+} is well known as a second messenger downstream of many cellular stimuli, including those that also modify endomembrane trafficking. From the examples outlined above it is clear that Ca^{2+} is a potent regulator of endomembrane trafficking, making it tempting to speculate that Ca^{2+} connects stimulus perception to modulation of endomembrane trafficking. Thus resolving the molecular mechanisms by which Ca^{2+} controls endomembrane trafficking remains one of the major open questions in plant cell biology.

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