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Update

The signatures of organellar calcium

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Abstract

Recent insights about the transport mechanisms involved in the in and out of calcium ions in plant organelles, and their role in the regulation of cytosolic calcium homeostasis in different signaling pathways.

Introduction

Plants are continuously subjected to environmental changes, such as fluctuating light, the day/night cycle, oscillating temperatures, water availability, and interaction with other organisms. Often these variations can be adverse, being stressful and thus affecting plant growth, development and, in several cases, causing major yield losses in agriculture. Especially, plants cannot move to a more comfortable environment to survive and they, thus, need to continuously monitor, and possibly anticipate, any upcoming stress (Zhu, 2016).

Plants' early responses to stress often occur in a time frame of seconds or a few minutes in different cell compartments, and in these cases, they mainly rely on quick changes of ions' concentrations (e.g. Ca^{2+}, H^{+}, K^{+}, NO^{3-}, Cl^{-}) which are dependent upon their movements across membranes (Stephan et al., 2016; Behera et al., 2018; Costa et al., 2018; Demes et al., 2020; Martí Ruiz et al., 2020). Among the ions, it is well-accepted that calcium (Ca^{2+}) plays a key role in many signal transduction pathways (Trewavas and Malhó, 1998; Sanders et al., 2002; Dodd et al., 2010; Kudla et al., 2010, 2018; Tian et al., 2020). At rest, in plant cells, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is in the range of 100–200 nM (Trewavas, 1999; Logan and Knight, 2003; Stael et al., 2012; Jezek and Blatt, 2017), but it can rise steeply (often too low μM concentrations) in response to the perception of different stimuli (Knight et al., 1997; Ranf et al., 2008). Importantly, the stimulus-induced increase in $[Ca^{2+}]_{cyt}$ is not a "digital signal," but an "analog" one that, depending on the stimulus type and magnitude, assumes a peculiar dynamic often dubbed "cytosolic Ca^{2+} signature" (Sanders et al., 2002; Kudla et al., 2010, 2018; Tian et al., 2020). The model predicts that different signatures activate different Ca^{2+} sensors (e.g. Calmodulin [CaM], CaM-like proteins [CMLs], calcineurin β-like proteins [CBLs]), and Ca^{2+} relays (e.g. Ca^{2+}-dependent protein kinases [CDPKs], or CaM-dependent protein kinase [CCaMK]) that trigger precise and tailored responses, altering gene expression and metabolism (Figure 1; reviewed in Sanders et al., 2002; McCormack et al., 2005; DeFalco et al., 2009; Hamel et al., 2014; Edel et al., 2017; Kudla et al., 2018; Lenzoni et al., 2018; Poovaiah and Du, 2018; Liu et al., 2020; Tang et al., 2020). The understanding of how the Ca^{2+} signature is generated and shaped to assume its "analog nature" is a key aspect to dissect the roots of Ca^{2+} signaling, which is traceable to its movement across membranes. Thus, important questions are (1) how do plant cells generate specific Ca^{2+} signatures? (2) which are the mechanisms for their tight regulation? A simple answer is that generation and shaping of cytosolic

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ADVANCES

- Genetically encoded sensors allow the in vivo analysis of Ca^{2+} dynamics in different subcellular compartments.
- Clear in vivo evidence demonstrated that subcellular compartments accumulate and release Ca^{2+} ions.
- The alteration of Ca^{2+} transport in the subcellular compartments alters cytosolic Ca^{2+} signatures with downstream effects on gene regulation and sensitivity to stress.
- Direct and indirect evidence supports the role of the ER as both a sink and source of Ca^{2+}.
- Indirect evidence supports the role of the vacuole as both a sink and source of Ca^{2+}.

Ca^{2+} signatures is dependent on a coordinate and intertwined activity of Ca^{2+} permeable channels, Ca^{2+} transporters, and Ca^{2+} buffers (Sanders et al., 2002; McAinsh and Pittman, 2009; Michard et al., 2011; Costa et al., 2017; Behera et al., 2018; Wudick et al., 2018; Hilleary et al., 2020; Tian et al., 2020). Whereas it is reported that the major source of cytosolic Ca^{2+} is the apoplast and that the movement of Ca^{2+} across the plasma membrane (PM) plays a major role in the generation of cytosolic Ca^{2+} transients (Gao et al., 2004; Stael et al., 2012; Costa et al., 2018; Tian et al., 2019; Lopez-Hernandez, 2020; Tian et al., 2020), increasing evidence demonstrates that subcellular compartments are involved in the shaping of the signatures, working as Ca^{2+} sinks (Qudeimat et al., 2008; Costa et al., 2010; Loro et al., 2012; Nomura et al., 2012; Bonza et al., 2013; Nomura and Shiina, 2014; Wagner et al., 2015; Loro et al., 2016; Lenglet et al., 2017; Behera et al., 2018; Corso et al., 2018, Teardo et al., 2019; Hilleary et al., 2020), as well as Ca^{2+} sources (Wang et al., 2010; Zhu et al., 2010; Tian et al., 2014, Shkolnik et al., 2018). Interestingly, some compartments (i.e. chloroplast and nucleus) can also generate their own organellar Ca^{2+} signature (Xiong et al., 2004; Loro et al., 2016; Sello et al., 2016; 2018; Kelner et al., 2018; Leitão et al., 2019) adding a additional level of complexity to Ca^{2+} signaling (Figure 1).

In this update, we will provide readers with an evidence that subcellular compartments experience their own Ca^{2+} transients in response to different stimuli, with particular attention to describing those cases where their role in the shaping of cytosolic Ca^{2+} dynamics and in the regulation of downstream responses was experimentally observed. We will give examples to demonstrate that cytosolic and organellar Ca^{2+} are directly intertwined, as well as that dysfunctions in the transport of other ions can lead to altered cytosolic Ca^{2+} signatures and visible phenotypes. For simplicity, we will divide our update into four different fields of cellular signaling: (1) plant responses to abiotic stimuli, (2) plant responses to biotic stimuli, (3) stomatal movements, and (4) plant developmental processes. We will not discuss the chloroplast Ca^{2+} signatures observed in responses to light–dark transitions and in response to heat stress (Sai and Johnson, 2002; Loro et al., 2016; Sello et al., 2016; Frank et al., 2019; Lenzoni and Knight, 2019; Martí Ruiz et al., 2020), as those two mechanisms are extensively covered in the update by He et al. (2021) of this Focus Issue.

Contribution of organelles in the shaping of cytosolic calcium increases in response to salt and osmotic stresses

Plants are particularly sensitive to abiotic stress, and their perception elicits (1) characteristic $[Ca^{2+}]_{cyt}$ increase, (2) activation of protein kinases and phosphatases, (3) modulation of gene expression, and (4) hormone biosynthesis (Gong et al., 1998; van Der Luit et al., 1999; Knight and Knight, 2001; Zhu, 2002; 2016; Tracy et al., 2008; Peleg and Blumwald 2011; Krebs et al., 2012; Yuan et al., 2014; Choi et al., 2014a, 2014b; Wagner et al., 2019). In this section, we will detail recent evidence showing the involvement of different subcellular compartments in the shaping of cytosolic Ca^{2+} signature in response to abiotic stress, focusing on salt and osmotic stress, and reporting, when available, the consequent downstream effects on the plant's physiology.

Salt and osmotic stresses are stimuli that induce in plants a quick $[Ca^{2+}]_{cyt}$ increase with dose-dependent as well as tissue-specific responses (Kiegle et al., 2000). The application of these stresses has been efficiently exploited to design genetic screenings that were instrumental in identifying salt- and osmosensors as well as key components involved in the signal transduction (Yuan et al., 2014; Jiang et al., 2019; Chen et al., 2020). Such genetic screenings were based on Arabidopsis (*Arabidopsis thaliana*) seedlings expressing the genetically encoded Ca^{2+} sensor aequorin targeted to the cytosol (Box 1; Figure 2). Moreover, the exposure to salt and osmotic stress of Arabidopsis seedlings expressing genetically encoded Ca^{2+} sensors localized in different subcellular compartments (Box 1; Figure 2; reviewed in Stael et al., 2012; Costa et al., 2018), highlighted that besides the cytosol, also chloroplasts, nongreen plastids, mitochondria, endoplasmic reticulum (ER), Golgi apparatus (GA), and nuclei experience Ca^{2+} transients (Xiong et al., 2004; Loro et al., 2012; Ordenes et al., 2012; Bonza et al., 2013; Sello et al., 2016; Huang et al., 2017; Corso et al., 2018; Kelner et al., 2018; Teardo et al., 2019). In most of the reported cases, the analyzed subcellular compartments showed Ca^{2+} transients that followed the $[Ca^{2+}]_{cyt}$ increase, therefore, behaving as sinks and not as sources of Ca^{2+} (reviewed in Stael et al., 2012; Costa et al., 2018). This result allows us to hypothesize a primary role for subcellular compartments in the shaping of the cytosolic Ca^{2+} signature rather than in its generation (McAinsh and Pittman, 2009).

Organellar calcium in salt stress responses

Plants expressing genetically-encoded Ca^{2+} indicators (GECIs) targeted to different subcellular compartments (i.e.

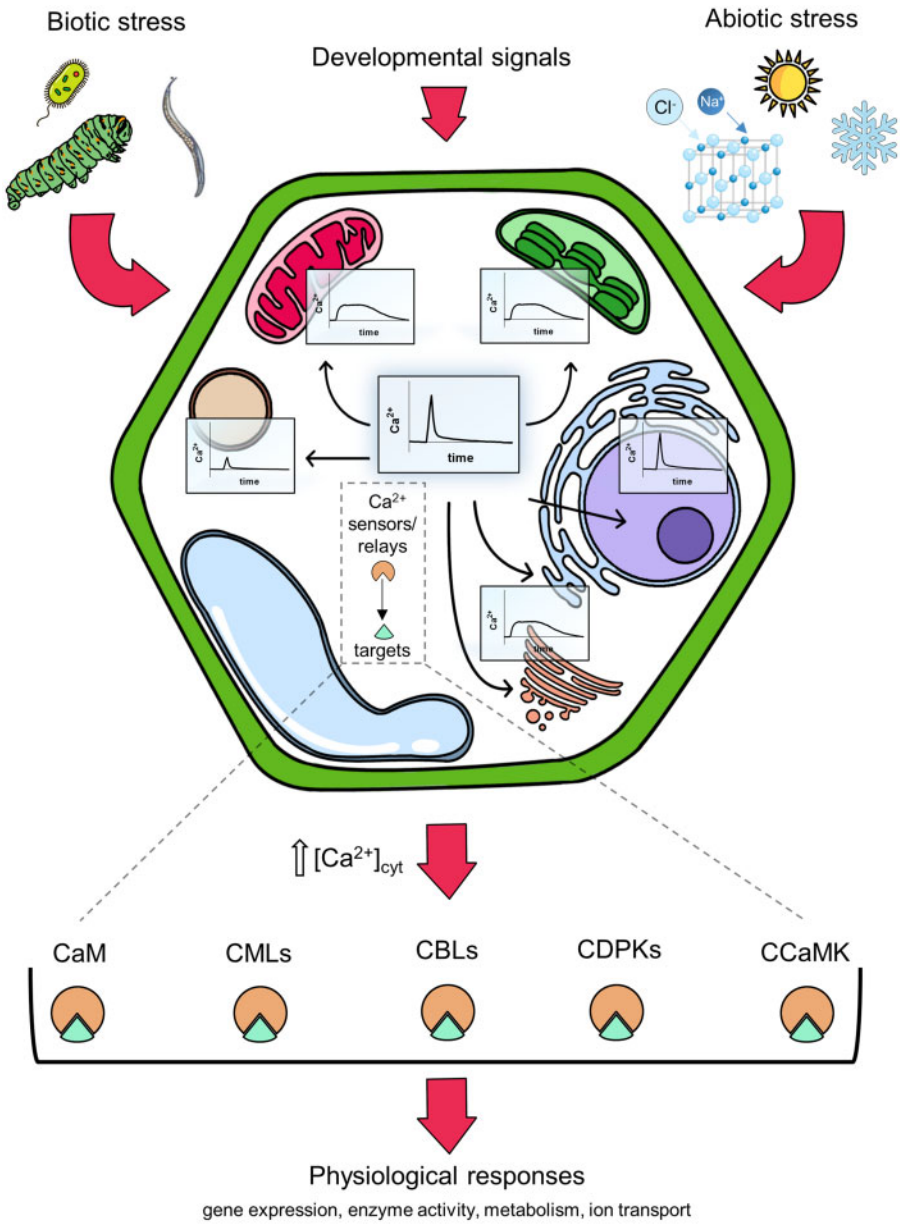


Figure 1 Biotic and abiotic stress as well as developmental stimuli can trigger cytosolic and organellar [Ca^{2+}] increases. Cytosolic Ca^{2+} transients are decoded by different Ca^{2+} sensors and relays, such as CaM, CMLs, CBLs proteins, CDPKs, and CCaMK that trigger precise and tailored responses altering gene expression and metabolism.

aequorin, Cameleon, R-GECO1, and G-GECO1.2; Knight et al., 1991; Miyawaki et al., 1997; Zhao et al., 2011; Box 1; Figure 2) were instrumental to show that salt stress induces a transient accumulation of Ca^{2+} in the cytosol, ER, chloroplasts/plastids and nucleus (Bonza et al., 2013; Sello et al., 2016; Huang et al., 2017; Corso et al., 2018; Kelner et al., 2018; Teardo et al., 2019). Some pieces of evidence support the role of ER, chloroplasts, and vacuoles in the dampening and/or shaping of the cytosolic Ca^{2+} increase.

The genetic ablation of the ER-localized Arabidopsis Ca^{2+}/cation transporter AtCCX2 (*ccx2*-knockout [KO] mutant) leads to a reduced salt-induced $[Ca^{2+}]_{cyt}$ increase, and less salt-tolerant plants compared to wild-type. Remarkably, *CCX2* overexpression (*CCX2*-OX) has the opposite effect, with an exacerbation of the $[Ca^{2+}]_{cyt}$ transient maximum and plants more tolerant to salt stress compared to wild-type (Corso et al., 2018). Thus, a link between the magnitude of the Ca^{2+} signature and plant tolerance was proposed. These data highlight the involvement of AtCCX2 and, therefore, of the ER, in the regulation of cytosolic Ca^{2+} in response to salt stress (Corso et al., 2018). The study of AtCCX2 topology in the ER membrane, as well as Ca^{2+} transport analyses in isolated ER fractions from wild-type and mutant plants, should allow us to better

BOX 1 Genetically encoded Ca^{2+} indicators

Genetically Encoded Calcium Indicators (GECIs) allow a non-invasive monitoring of free Ca^{2+} concentrations [Ca^{2+}] in different subcellular compartments. In plants, the two mainly used GECIs have been aequorin and Cameleon.

Aequorin enables monitoring of Ca^{2+} dynamics by photon emission measurements in transformed plants after reconstitution of the aequorin holoenzyme with the exogenously applied prosthetic group coelenterazine (Knight et al., 1991). Due to its low quantum yield, aequorin suffers from poor spatial resolution. Aequorin has been targeted to the cytosol, nucleus, endoplasmic reticulum (ER), the Golgi apparatus, mitochondria, chloroplasts, and apoplast (reviewed in Costa et al., 2018). To improve the spatial resolution the use of fluorescent Ca^{2+} sensors was exploited. The ratiometric Ca^{2+} reporter Cameleon was the first fluorescent GECI expressed in plant cells (Allen et al., 1999). Cameleon exploits the Förster Resonance Energy Transfer (FRET) property occurring between one fluorescent protein that acts as a donor (e.g. CFP) that, when excited, transfers absorbed energy to a second fluorescent protein, the acceptor (e.g. YFP or cpVenus). The efficiency of FRET depends on the donor-acceptor distance which is dependent upon the CaM-M13 Ca^{2+}-dependent interaction. The CaM-M13 sensor is sandwiched between the two fluorophores (Miyawaki et al., 1997). With Cameleon, the readout is the ratio between acceptor and donor fluorescence emissions which reduces artefacts due to the expression level of the sensor or focus changes. Cameleon has been targeted to the cytosol, nucleus, ER, mitochondria, chloroplasts, and peroxisomes (Costa et al., 2018).

Intensiometric GFP-based Ca^{2+} sensors have also been developed and successfully used in plants. We can cite GCaMP3, GCaMP6, and the green and red variants of GECO1 or CEPIA (Zhao et al., 2011; Costa et al., 2018; Kelner et al., 2018; Luo et al., 2020). All these GECIs rely on a change in the sensor quantum yield, measured as a change of the intensity of the emitted fluorescence which depends upon the amount of Ca^{2+} bound to the sensory domain. A change of [Ca^{2+}] affects the CaM conformation which is transmitted to a circularly permuted variant of green (e.g. cpGFP) or red (e.g. cpmApple) fluorescent proteins. Intensiometric biosensors exhibit a higher signal change compared to a FRET-based sensor, but a change of their expression level could be misinterpreted as a change in free Ca^{2+} concentration (Costa et al., 2018). GCaMPs, R-GECO1 and R-CEPIA have been targeted to the cytosol, nucleus, and ER (Kelner et al., 2018; Luo et al., 2020).

define the AtCCX2 mode of action and its physiological function.

The role of chloroplasts in the regulation of $[Ca^{2+}]_{cyt}$ increase in response to salt stress (e.g. NaCl) was also demonstrated. This evidence came from a genetic screen of Arabidopsis mutants defective in salt stress-induced $[Ca^{2+}]_{cyt}$ elevation that allowed the identification of the chloroplast-localized glycosyl-transferase QUASIMODO1 (AtQUA1), a protein involved in the stacking of thylakoids grana. Compared to the wild-type, the *qua1-4* mutant exhibited a dramatically greater increase in $[Ca^{2+}]_{cyt}$ under NaCl treatment (Zheng et al., 2017), and this effect was linked to the activity of the chloroplast Ca^{2+} sensor Calcium Sensing Receptor (CAS; Han et al., 2003; Nomura et al., 2008; 2012). There was no measurement of [Ca^{2+}] in the chloroplast stroma or thylakoid lumen of the *qua1-4* mutant, thus, we lack experimental evidence that the alteration of $[Ca^{2+}]_{cyt}$ is directly dependent on a deregulated chloroplast Ca^{2+} handling. Nevertheless, in independent studies, by using Arabidopsis plants expressing the Ca^{2+} sensor aequorin directed to the chloroplasts/plastids stroma (Box 1; Figure 2), it has been shown that these compartments transiently accumulate Ca^{2+} in response to salt stress (Sello et al., 2016; Teardo et al., 2019). At the present time, knowledge about the molecular players (Ca^{2+}-permeable channels or transporters in the outer and/or inner membrane) involved in the chloroplast Ca^{2+} accumulation in response to salt stress is rather limited. The Arabidopsis glutamate receptor-like channel AtGLR3.4 is permeable to Ca^{2+} (Vincill et al., 2012; Box 2; Figure 3), and besides being localized in the PM (Meyerhoff et al., 2005; Vincill et al., 2012) it was also reported to be present in chloroplasts (Teardo et al., 2011). Two Arabidopsis independent AtGLR3.4 mutant alleles, *atglr3.4-1* and *atglr3.4-2*, showed an impaired NaCl-induced $[Ca^{2+}]_{cyt}$ increase and a more salt-sensitive phenotype during seed germination and postgermination growth compared to wild-type plants (Cheng et al., 2018). However, the fact that data regarding the Ca^{2+} dynamics in the chloroplasts/plastids of these mutants are lacking, does not allow clear conclusions to be drawn. In addition, no other evidence has supported the chloroplast localization of GLR3.4, thus this result might require validation.

The role of intracellular compartments in the shaping of $[Ca^{2+}]_{cyt}$ transients in response to salt stress was also found in the moss *Physcomitrium patens* (formerly *Physcomitrella patens*), where the mRNA coding for the tonoplast-localized PIIB-type Ca^{2+}-ATPase (Box 2; Figure 3) PCA1 was shown to be upregulated by NaCl treatment. *PCA1* loss-of-function mutants (Δ*PCA1*) exhibit a sustained elevated $[Ca^{2+}]_{cyt}$ in response to salt, and an enhanced salt susceptibility and higher cell death rates upon treatment. Remarkably, the altered Ca^{2+} response in the Δ*PCA1* lines corresponded with the altered expression level of stress-induced genes, suggesting the disturbance of a stress-associated signaling pathway

(Qudeimat et al., 2008). Although direct evidence of an impaired Ca^{2+} accumulation in the vacuole in the Δ*PCA1* was not reported, the well-defined role of PIIB-type Ca^{2+}-ATPases in the transport of Ca^{2+} out of the cytosol (Box 2) strongly supports a direct role of PCA1 in the restoration of prestimulus $[Ca^{2+}]_{cyt}$, with the vacuole working as a cytosolic Ca^{2+} capacitor/buffer (Qudeimat et al., 2008). In Arabidopsis, the two tonoplast-localized PIIB-type Ca^{2+}-ATPase isoforms AtACA4 and AtACA11 (Box 2, Figure 3) have a role in the regulation of $[Ca^{2+}]_{cyt}$ in leaf epidermal cells in response to bacterial flagellin (flg22; see next section; Hilleary et al., 2020), and in the salicylic acid (SA)-dependent programmed cell death pathway (Boursiac et al., 2010), but so far, in plants, no data are available regarding their role in salt stress, even if ACA4 conferred protection against osmotic stress such as high NaCl, KCl, and mannitol when expressed in yeast cells (Geisler et al., 2000). Nevertheless, a key role of the vacuole in the regulation of $[Ca^{2+}]_{cyt}$ in response to this stress was shown in Arabidopsis. Choi et al. (2014b), by challenging Arabidopsis seedling root tip cells with 100 mM NaCl, demonstrated the existence of a long-distance root-to-shoot cytosolic Ca^{2+} wave responsible for the induction of the expression of stress-regulated genes in the shoot. The role of the vacuole in this process was evidenced by the fact that the ablation of the tonoplast-localized two-pore channel AtTPC1 (Peiter et al., 2005) strongly dampened the speed of the Ca^{2+} wave, whereas its overexpression boosted it (Choi et al., 2014b). Importantly, upon salt stress, the *tpc1*-KO mutant did not show the upregulation of stress-related marker genes in the shoot such as the Multi-Stress-Responsive Zinc-Finger Protein (*ZAT12*) and the CaM-Related Touch 2 (*TCH2*; Choi et al., 2014a, 2014b). Despite some controversy about a direct involvement of AtTPC1 in the release of Ca^{2+} from the vacuole (Ranf et al., 2008; Beyhl et al., 2009; Gradogna et al., 2009; Lenglet et al., 2017, Hedrich et al., 2018, Jaślan et al., 2019), these results demonstrate the importance of this compartment in the plant response to salt stress and in the overall strategy that plants adopt to cope with it. Of note, Dindas et al. (2021) have recently proposed, through a modeling approach, that the involvement of AtTPC1 in the long-distance signaling is not due to its direct involvement in the release of Ca^{2+} from the vacuole, but rather depends on its capacity to transport K^+ which is regulated by the vacuolar membrane potential. Thus, in accordance with Jezek and Blatt (2017), the cytosolic Ca^{2+} signature could be intimately connected with the flux of ions other than Ca^{2+}.

Defining the importance of the subcellular compartments in the shaping of cytosolic Ca^{2+} signatures in response to salt stress was achieved by an orthogonal approach. Thanks to the expression, in Arabidopsis plants, of the rat Ca^{2+}-binding protein parvalbumin (PV) fused to either a nuclear export (PV-NES) or a nuclear localization sequence (NLS-PV), the cytosolic or nucleosolic Ca^{2+} were selectively buffered (Huang et al., 2017). When both cytosolic and nuclear Ca^{2+} levels were monitored in root cells by means of the Cameleon Ca^{2+} sensor (YC3.6; Box 1; Figure 2), both compartments experienced salt- and osmotic-induced $[Ca^{2+}]$ increases. Buffering of nuclear Ca^{2+} prevented the nuclear Ca^{2+} increase but not the cytosolic one, whereas buffering of cytosolic Ca^{2+} did not affect the nuclear Ca^{2+} rise. In both cases, in response to salt stress, the length of the primary root was impaired, and the expression of several abiotic stress-induced genes was deregulated, especially when nuclear Ca^{2+} was buffered (Huang et al., 2017). This latter result strongly supports the idea that the alteration of cellular Ca^{2+} signatures, in particular at the nuclear level, has a direct effect on the regulation of gene transcription and is

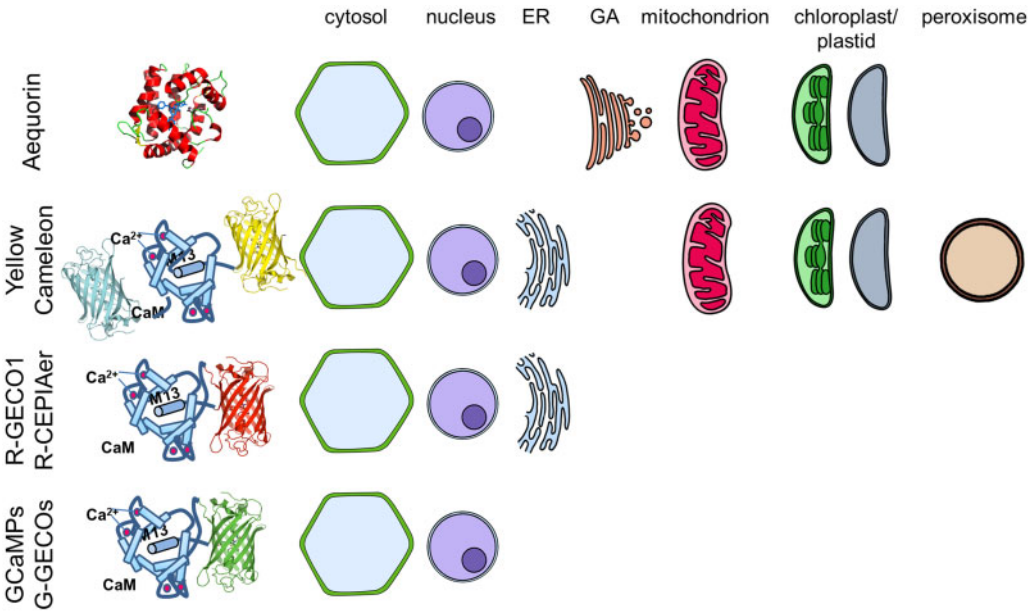


Figure 2 Overview of organelle-targeted genetically encoded Ca^{2+} indicators.

BOX 2 Ca^{2+} TRANSPORT SYSTEMS IN PLANTS.

Even though plants do not possess canonical Ca^{2+} channels, they are equipped with Ca^{2+}-permeable channels that establish a hydrophilic path for Ca^{2+} diffusion down its electrochemical gradient. Different Ca^{2+}-permeable channel families are localized at the PM, facilitating Ca^{2+} influx from the apoplast, but can be also resident at the inner membranes, such as those surrounding the ER, GA, mitochondria, chloroplasts, vacuole, and possibly peroxisomes.

Ca^{2+}-extruding systems actively remove Ca^{2+} from the cytosol either by transporting it out of the cell across the PM or into internal organelles. These transporters include Ca^{2+} pumps and Ca^{2+}/cation antiporters (CaCAs).

Ca^{2+}-ATPases are the major active transport system that ensures the compartmentalization of Ca^{2+}. They are members of the P-type ATPases superfamily that use the energy of ATP hydrolysis to pump Ca^{2+} from the cytoplasm to the apoplast or into intracellular compartments. Plant cells possess two types of Ca^{2+}-pumping ATPase, belonging to subgroups PIIA-type (ER-type Ca^{2+}-ATPase) and PIIB-type (auto-inhibited Ca^{2+}-ATPase) that have been found at the tonoplast, PM, ER, Golgi, nucleus, and perhaps also at the plastid envelope. While the autoinhibitory regulative mechanism of PIIB-type members has been extensively studied and characterized, very little is known about the regulation of those belonging to PIIA-type subfamily in plants.

The CaCA superfamily comprises cation/Ca^{2+} exchangers (CCX) and Ca^{2+}/proton exchangers (CAX) that derive the energy needed to mediate active Ca^{2+} transport from the electrochemical gradient of protons across membranes facing the cytosol.

While Ca^{2+} pumps are high affinity and low-capacity systems, exchangers are characterized by low-affinity but high-capacity Ca^{2+} sequestration. The coordinated action of both extrusion mechanisms covers a wide range of physiological cytosolic Ca^{2+} concentrations.

A detailed analysis of different Ca^{2+} transport mechanisms across plant membranes has been extensively reviewed in Demidchik et al. (2018).

in accordance with the demonstration that Ca^{2+} can regulate gene expression via CDPK and CaM binding transcription factors (CAMTA; Reddy et al., 2011; Dubiella et al., 2013; Gao et al., 2013; Whalley and Knight, 2013; Lenzoni and Knight, 2019; Liu et al., 2020).

Organellar calcium in osmotic stress responses

Salt imposes on plant cells both ionic and osmotic stress, but this latter is per se detrimental for plant growth (Xiong and Zhu, 2002; Yang and Guo, 2018). Both hyperosmotic and hypoosmotic stress elicit $[Ca^{2+}]_{cyt}$ increases (Yuan et al., 2014; Basu and Haswell, 2020). Similar to what occurred with salt stress (see above), the Arabidopsis *ccx2*-KO or *CCX2*-OX plants showed a smaller and a higher $[Ca^{2+}]_{cyt}$ increase respectively compared to the wild-type when subjected to both hyperosmotic and hypoosmotic shocks. Moreover, in response to the same stress, the ER of the *ccx2*-KO accumulated more Ca^{2+} than the wild-type. Overall, these data demonstrate that the AtCCX2 transporter is involved in the regulation of Ca^{2+} transport between the ER and cytosol (Figure 3) in response to both hyperosmotic and hypoosmotic stress (Corso et al., 2018). Interestingly, even if localized to the tonoplast, the *Oryza sativa* (rice) CCX2 (OsCCX2; Figure 3) that mediates Ca^{2+}/cation transport in yeast, is also upregulated in response to drought stress (Yadav et al., 2015). However, at present, no information about its putative role in the regulation of intracellular Ca^{2+} homeostasis has been reported.

Being triggered by a drop in the soil water potential, hydrotropism can be considered as a response to a sort of osmotic stress (Dietrich, 2018; Fromm, 2019). The involvement of the ER in the regulation of Ca^{2+} signaling was recently highlighted in the hydrotropic response of Arabidopsis roots. The inhibition of AtECA1, a PIIA-type Ca^{2+}-ATPase (Box 2; Figure 3), by the Myc-Interacting Zinc-Finger Protein 1 (AtMIZ1) determined a slow asymmetric rise of $[Ca^{2+}]_{cyt}$ in the elongation zone of root tip cells which was required for the root bending toward areas of higher water potential. The Arabidopsis *eca1*-KO and the pharmacological inhibition of AtECA1 by cyclopiazonic acid in wild-type seedlings produced a higher $[Ca^{2+}]_{cyt}$ increase and exacerbated the root bending response, demonstrating the key role of the ER in this signaling pathway (Shkolnik et al., 2018). Remarkably, by using Arabidopsis seedlings expressing an ER-localized Cameleon Ca^{2+} sensor (CRT-D4ER; Bonza et al., 2013; Box 1; Figure 2), it was shown that the hydrotropic stimulus triggered a decrease in ER luminal $[Ca^{2+}]$, supporting a role for the ER as a Ca^{2+} source in this process (Shkolnik et al., 2018). This latter result is thus a demonstration of direct involvement of the ER in the generation of a $[Ca^{2+}]_{cyt}$ transient through the inhibition of a Ca^{2+} pump and not through the opening of Ca^{2+}-permeable channels. A better understanding of how PIIA-type Ca^{2+}-ATPases are fine-tuned is a key issue that will deserve attention in the near future.

A recent report demonstrated that the lack of the so-called AtcMCU, a member of the mitochondrial calcium

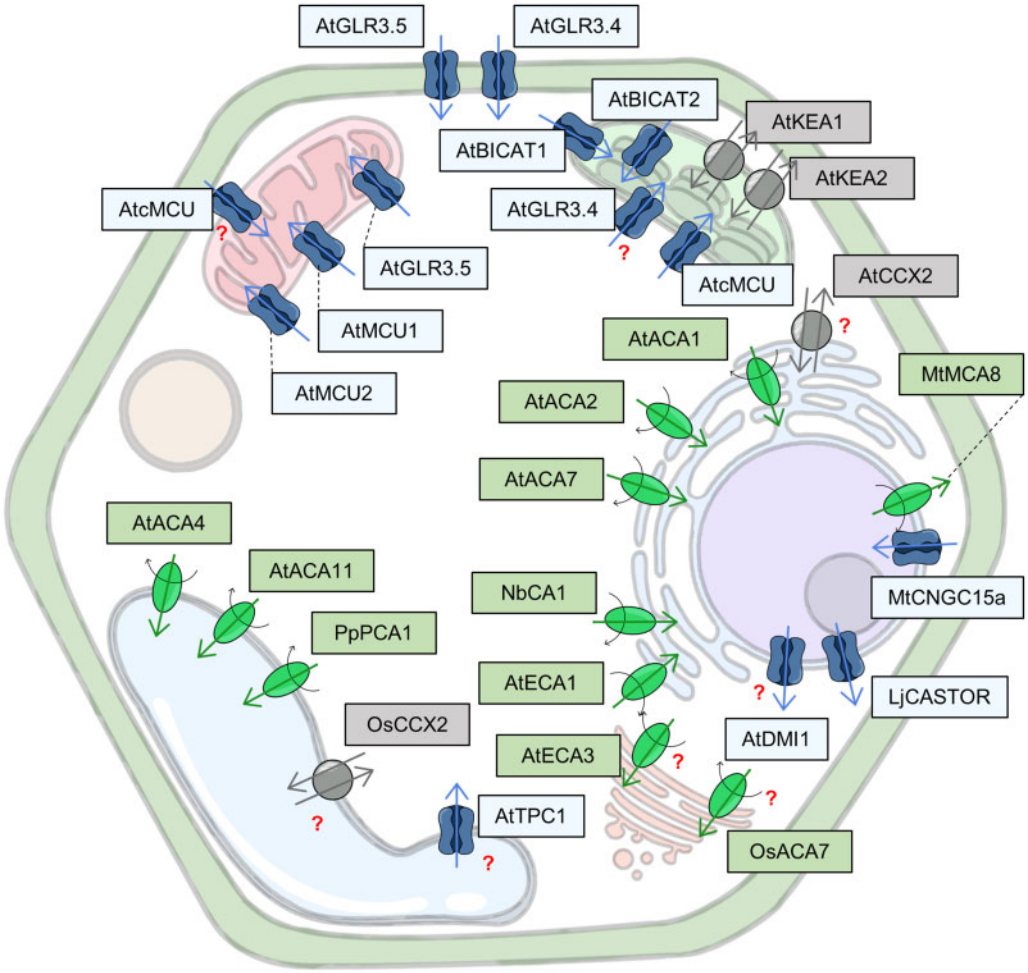


Figure 3 Channels and transporters localized in subcellular compartments reported to have a role in plant developmental processes and stress responses linked to the regulation of Ca^{2+} transport across membranes. The red question marks indicate that further studies are required. Channels are represented in blue, pumps in green, and cotransporters in grey. At, *Arabidopsis thaliana*; Lj, *Lotus japonicus*; Mt, *Medicago truncatula*; Nb, *Nicotiana benthamiana*; Pp, *Physcomitrium patens*; Os, *Oryza sativa*.

uniporter (MCU) family (Baughman et al., 2011; De Stefani et al., 2011; Stael et al., 2012; Box 2; Figure 3) localized both in mitochondria and chloroplasts (Teardo et al., 2019), reduced the stromal Ca^{2+} accumulation in response to hyperosmotic stress, with a consequent higher rise of the $[Ca^{2+}]_{cyt}$ peak compared to the wild-type (Teardo et al., 2019). The lack of activation of mitogen-activated protein kinase 3 and 6 (MAPK3/6) and a deregulated gene expression pattern in the *cmcu*-KO plants, suggested the hypothesis that AtcMCU is required for a retrograde chloroplast signaling in response to the osmotic stress (Teardo et al., 2019). On the other hand, the AtcMCU presence in mitochondria (Teardo et al., 2019) would suggest that, in the *cmcu*-KO, the hyperosmotic induced Ca^{2+} dynamics could be altered also in this compartment, but at the present time, data to support this hypothesis are lacking. Interestingly, AtcMCU did not seem to be involved in the transport of Ca^{2+} into chloroplasts during salt stress thus suggesting its stress-specific role in the osmotic response (Teardo et al., 2019).

The possibility that plastids/chloroplasts represent a hub for the regulation of cytosolic Ca^{2+} dynamics in response to osmotic stress is sustained by another recent study as well. The lack of two plastidial envelope-localized K^+ exchange antiporters (KEA) , AtKEA1 and AtKEA2 (Figure 3), impaired the rapid hyperosmotic-induced $[Ca^{2+}]_{cyt}$ increase (Stephan et al., 2016). At present, the mechanism responsible for the alteration of the cytosolic Ca^{2+} signature in the *kea1/kea2* mutant is not understood, and a study of stromal Ca^{2+} dynamics is required.

Based on the cited works, it is evident that ER, vacuole, and chloroplasts/plastids are involved in the response to different abiotic stresses by contributing to shaping the cytosolic Ca^{2+} dynamics, and that proper nuclear Ca^{2+} dynamics are important for the regulation of gene transcription. Despite the recent identification of some molecular players involved in the Ca^{2+} accumulation in mitochondria (Wagner et al., 2015; Teardo et al., 2017; Selles et al., 2018), and the proposed role of this organelle in the salt stress response through retrograde signaling (Vanderauwera et al.,

2012), there are no direct demonstrations that an impaired Ca^{2+} accumulation in this organelle can affect the cytosolic Ca^{2+} signature in response to abiotic stress.

Even less is known about a possible role of the other compartments, such as the GA, where the Arabidopsis PIIA-type Ca^{2+}-ATPase AtECA3 and the rice PIIB-type Ca^{2+}-ATPase OsACA7 have been localized (Figure 3; Mills et al., 2008; Singh et al., 2014). Interestingly, yeast cells expressing the OsACA7 are less sensitive to salt tolerance (Singh et al., 2014). However, since a clear role of these two pumps in planta has not been reported, further research in this direction is required.

Contribution of organelles in the shaping of cytosolic calcium increases in responses to biotic stimuli

One of the earliest events occurring in different plant biotic interactions is a rise in cytosolic and nuclear $[Ca^{2+}]$ (Blume et al., 2000; Oldroyd and Downie, 2006). Plant biotic interactions can be beneficial, as in the case of symbiosis with Rhizobia and Mycorrhiza, or detrimental as in those with a wide range of pathogens (e.g. *Pseudomonas syringae*). In this section, we will briefly touch on the role played by the nuclear Ca^{2+} in symbiotic interactions, and then we will address more extensively recent evidence of how organellar Ca^{2+} homeostasis is involved in the regulation of cytosolic Ca^{2+} signatures in response to pathogen elicitors.

Symbiotic interactions have been mainly studied in Leguminosae in which a clear role of nuclear Ca^{2+} signaling was reported (reviewed in Oldroyd and Downie, 2006; Charpentier, 2018). It is well known that the perception of nodulation (NOD) and mycorrhizal (MYC) factors trigger characteristic nuclear Ca^{2+} oscillations that are then transduced by the nuclear-localized Ca^{2+}- and CaM-dependent Ser/Thr protein kinase (CCaMK; reviewed in Charpentier and Oldroyd, 2013). As the symbiosis interaction topic and the role played by nuclear Ca^{2+} is so vast, we redirect the readers to a recent review (e.g. Charpentier, 2018). Nonetheless, it is worth noting that one of the major discoveries in the field, made by Charpentier et al. (2016), was the identification of the *Medicago truncatula* cyclic nucleotide-gated channel 15 (MtCNGC15) as one of the Ca^{2+}-permeable channels involved in the generation of NOD-induced nuclear Ca^{2+} oscillations (Box 2; Figure 3). Of note, MtCNGC15, being specifically localized in the nuclear envelope (NE), presumably promotes the release of Ca^{2+} from the ER directly into the nucleoplasm (Charpentier et al., 2016) even if a Ca^{2+} depletion from NE was not reported. Previously, it was also demonstrated that the *Medicago* PIIA-type Ca^{2+}-ATPase MtMCA8 is targeted to the inner NE membrane (Figure 3) and is essential for symbiotic Ca^{2+} oscillations, suggesting its role in efficient Ca^{2+} reloading from the nucleoplasm (Capoen et al., 2011). A mathematical modeling approach predicted that besides the need for a Ca^{2+}-permeable channel (i.e. MtCNGC15) and a Ca^{2+}-ATPase (i.e. MtMCA8), the generation of nuclear Ca^{2+} oscillations requires the activity of a Ca^{2+}-activated K^+ channel to counterbalance the movement of positive charges. The Doesn't Make Infections 1 (MtDMI1) channel is permeable to K^+, is localized at the NE, and is, therefore, presumably the component needed to counterbalance the charge caused by the influx of Ca^{2+} into the nucleoplasm (Granqvist et al., 2012). The same group later demonstrated that Ca^{2+} can diffuse from the nucleus into the cytosol (Kelner et al., 2018), but no physiological function has been ascribed to this $[Ca^{2+}]_{cyt}$ rise.

Pathogen-associated molecular patterns (PAMPs) are recognized by cell surface receptors (pattern-recognition receptors PRR) and activate an array of basal defense responses (PAMP-triggered immunity, PTI; Zipfel and Oldroyd, 2017; Zhang et al., 2020). In response to PAMPs, the plant immune system relies on two parallel signal transduction pathways based on MAPK and Ca^{2+} signaling leading to transcriptional reprogramming (Boudsocq et al., 2010). In Arabidopsis, the PAMP flagellin (flg22 peptide) induces a quick $[Ca^{2+}]_{cyt}$ increase in leaves and roots (Ranf et al., 2008; Yuan et al., 2017; Hilleary et al., 2020; Emonet et al., 2021). The flg22-induced $[Ca^{2+}]_{cyt}$ rise is suggested to be mediated by different classes of PM-localised Ca^{2+}-permeable channels with evidence supporting a role of both members of glutamate receptor-like channels (GLR) and cyclic nucleotide-gated channels (CNGC) families (Kwaaitaal et al., 2011; Tian et al., 2019; Bjornson et al., 2021). Nonetheless, in guard cells, the flg22-induced $[Ca^{2+}]_{cyt}$ increase is, at least partially, dependent on members of the reduced hyperosmolality-induced $[Ca^{2+}]_i$ increase channels (OSCA) family (AtOSCA1.3 and 1.7; Thor et al., 2020) suggesting that multiple families of Ca^{2+} channels contribute to the overall pattern-induced Ca^{2+} response in Arabidopsis.

Direct involvement of the subcellular compartments in the generation of the flg22-induced $[Ca^{2+}]_{cyt}$ has been predicted (Ma et al., 2017), but experimental evidence is still lacking. Nevertheless, by using Arabidopsis plants expressing chloroplast-localized aequorin, it was shown that they transiently accumulate Ca^{2+} in the stroma in response to flg22 and that this stromal Ca^{2+} transient temporally follows the $[Ca^{2+}]_{cyt}$ increase (Nomura et al., 2012). Similar to what was observed in response to salt stress (see the previous section), the lack of the chloroplast Ca^{2+} sensor AtCAS affected flg22-induced chloroplast Ca^{2+} dynamics, and such a CAS-dependent mechanism was involved in transcriptional reprogramming through retrograde signaling (Nomura et al., 2012). Neither clues about the mechanism of Ca^{2+} transport across chloroplasts' membranes, nor consequent impairment of the cytosolic Ca^{2+} signature were reported. The fact that the *cmcu*-KO had an impaired H_2O_2-induced chloroplast Ca^{2+} accumulation (Teardo et al., 2019) makes this channel a putative candidate to mediate the Ca^{2+} influx in response to flg22, but there is no direct evidence for this as yet.

The impairment of Ca^{2+} transport across the tonoplast was also shown to impact the cytosolic Ca^{2+} signature in

response to flg22 (Hilleary et al., 2020). The dissection of cytosolic Ca^{2+} dynamics by using the ultrasensitive Ca^{2+} sensor Cameleon YC-Nano 65 (Horikawa et al., 2010; Choi et al., 2014b) in epidermal leaf cells of Arabidopsis plants lacking the two tonoplast-localized PIIB-type Ca^{2+}-ATPase AtACA4 and AtACA11 (Figure 3), revealed that this double KO mutant exhibited higher basal $[Ca^{2+}]_{cyt}$ and a larger flg22-induced Ca^{2+} increase compared to the wild-type. This was accompanied by an upregulation of the defense regulator gene *CBP60g* and a higher resistance to *Pst* bacterial challenge at 1 d postinoculation (Hilleary et al., 2020). No attempts to measure vacuole Ca^{2+} dynamics were carried out. However, the *aca4/aca11* mutant phenotype was complemented when the PIIB-type PM localised-Ca^{2+}-ATPase AtACA8, was (mis)targeted to the tonoplast of the mutant. This latter result strongly supports the idea that the observed phenotype of *aca4/aca11* was directly dependent on the impairment of Ca^{2+} import into the vacuole lumen. Very similar observations were made in the Arabidopsis *aca1/aca2/aca7* triple KO mutant lacking three ER-localized PIIB-type Ca^{2+}-ATPases. In comparison to wild-type, the triple mutant showed (1) a higher $[Ca^{2+}]_{cyt}$ in leaf cells at rest, (2) an increased magnitude and duration of the $[Ca^{2+}]_{cyt}$ transient induced by flg22 treatment, (3) smaller rosettes, and (4) a high frequency of leaf lesions (Ishka et al., 2021). Similar to the *aca4/aca11* mutant, the lesions phenotype of *aca1/aca2/aca7* was suppressed by the expression of a transgene encoding NahG, an enzyme that degrades SA (Ishka et al., 2021). Once again these results demonstrate the importance of the fine-tuning of the cytosolic Ca^{2+} homeostasis both at rest and during the recovery of the prestimulus $[Ca^{2+}]_{cyt}$, a process mediated by active Ca^{2+} transporters present in the tonoplast and ER, extruding Ca^{2+} out of the cytosol (Qudeimat et al., 2008; Costa et al., 2017; Hilleary et al., 2020; Ishka et al., 2021; Box 2; Figure 3).

Besides Arabidopsis, *Nicotiana tabacum* plants have also been used as a model for several studies investigating the role of cytosolic and organellar Ca^{2+} in the response to pathogen elicitors. *N. tabacum* plants are particularly sensitive to cryptogein, a 10-kDa protein secreted by the oomycete *Phytophthora cryptogea*, that induces a hypersensitive response in *Nicotiana tabacum* (var. Xanthi) plants and systemic acquired resistance against various pathogens (Bourque et al., 2002). Experiments using cells of *Nicotiana tabacum* var. Xanthi expressing aequorin localized to the cytosol, chloroplast, and mitochondria showed that cryptogein was effective in inducing specific Ca^{2+} signatures in each compartment with chloroplasts clearly showing a Ca^{2+} increase that peaked after the cytosolic one (Manzoor et al., 2012). Interestingly, a pharmacological approach indicated that inositol trisphosphate (IP3) could play a role in the cryptogein-induced Ca^{2+} signaling, suggesting its possible contribution in promoting a Ca^{2+} release from intracellular Ca^{2+} stores (e.g. vacuole or ER; Manzoor et al., 2012). Indeed, the silencing of the *N. tabacum* ER-localised PIIB-type Ca^{2+}-ATPase NbCA1 (Figure 3) accelerated the cryptogein-induced cell death in leaf cells (Zhu et al., 2010). Importantly, by using a genetically encoded Ca^{2+} sensor (Case 12) the authors showed that the downregulation of NbCA1 resulted in an altered cryptogein-induced cytosolic Ca^{2+} signature, with an increase of amplitude and duration of Ca^{2+} spikes. These findings suggest that the NbCA1 is involved in the Ca^{2+} efflux pathway that controls cell death during plant innate immune response (Zhu et al., 2010). In addition, other proteinaceous elicitors including flg22 were shown to induce in *N. tabacum* cells, cytosolic, and nuclear Ca^{2+} elevations, with this latter apparently dependent on $[Ca^{2+}]_{cyt}$, IP3, and reactive active oxygen species (ROS; Lecourieux et al., 2005).

The involvement of Ca^{2+} transport across ER in plant-pathogen interactions was also reported by studying Os*XA10*, a *R* gene for resistance to bacterial blight in rice. The OsXA10 protein is localized at the ER membrane where it is assembled as hexamers potentially working as a Ca^{2+} transporter (Tian et al., 2014). It was shown that the expression of OsXA10 in different systems (e.g. rice, *Nicotiana benthamiana*, and mammalian HeLa cells) triggered programmed cell death in a mechanism that is dependent on the release of Ca^{2+} from the ER (Tian et al., 2014). In leaf epidermal cells of *N. benthamiana*, the expression of the OsXA10-mCherry fusion disturbed the Ca^{2+} homeostasis, inducing a Ca^{2+} depletion of the ER accompanied by a $[Ca^{2+}]_{cyt}$ increase. Similar results were also obtained by expressing the OsXA10-RFP fusion in human HeLa cells (Tian et al., 2014). Noticeably, the expression of OsXA10 variants shown to be able to abolish the ER Ca^{2+} depletion also abolished the programmed cell death in *N. benthamiana* and HeLa cells as well as the disease resistance in rice (Tian et al., 2014) strongly supporting, in this specific case, the role of ER as a source of Ca^{2+} in the triggering of a $[Ca^{2+}]_{cyt}$ increase and promoting cell death.

Whereas the OsXA10 protein has an apparent direct role in the plant response to pathogens through the regulation of Ca^{2+} homeostasis in the ER, other indirect evidence highlighted the importance of proper Ca^{2+} homeostasis of this intracellular compartment in response to biotic stress. In Arabidopsis, the ER-localized Ca^{2+} binding protein calreticulin 3 (AtCRT3) is essential for the correct maturation of the EF-Tu receptor (EFR; Saijo et al., 2009) which recognizes the bacterial elicitor elf18 on the cell surface (Zipfel et al., 2006). Although Ca^{2+} measurements in the ER lumen of the At*crt3* mutant are lacking, this result provides indirect evidence about the critical role of ER Ca^{2+} homeostasis in plant immunity.

In conclusion, the involvement of ER, vacuole, and chloroplasts in the regulation of cytosolic Ca^{2+} homeostasis/Ca^{2+} transients induced in response to pathogen elicitors has been clearly demonstrated. In contrast, even if *N. tabacum* plants treated with cryptogein showed an accumulation of Ca^{2+} in the mitochondrial matrix (Manzoor et al., 2012), evidence of a role for mitochondrial Ca^{2+} elevations in this physiological context is still missing. However, the fact that

a splice variant of the Arabidopsis AtGLR3.5 was localized to the inner mitochondrial membrane and that the *glr3.5* mutant showed accelerated leaf senescence (Teardo et al., 2015), allows speculation about a role of mitochondrial Ca^{2+} transport in the plant responses to biotic stress. On the other hand, the *glr3.5* showed a mild phenotype regarding the mitochondrial Ca^{2+} uptake in response to leaf wounding and no mutant showing a strong impairment in the transport of Ca^{2+} has been reported yet. The isolation of such a line would represent a good tool to further investigate any possible role of this compartment in plant-pathogen interactions as well as other responses.

Contribution of organelles in the regulation of calcium homeostasis in stomatal movements

Stomata are made of a pair of guard cells present mainly in the leaf epidermis, and through them, plants acquire the CO_2 needed for photosynthesis and release O_2. When stomatal pores are open, plants, besides exchanging gases, lose water via transpiration, and, therefore, to prevent an excessive loss of water with consequent plant wilting, the opening of the pores must be finely controlled (Taiz et al., 2014).

Stomata open in response to light and close in response to drought stress, elevated CO_2, ozone, low humidity, pathogen attack, and other stimuli (reviewed in Kim et al., 2010). The importance of stomata in plant physiology has elected guard cells as a highly developed model system for dissecting signal transduction mechanisms in plants, and for elucidating how individual signaling mechanisms can interact within a network in a single cell (Kim et al., 2010). Indeed, stomatal guard cells represent, together pollen tubes and root hairs (see below) cell models in which Ca^{2+} signaling has been deeply investigated (reviewed in Kim et al., 2010; Jezek and Blatt, 2017).

The opening and closing of stomata are mechanistically regulated by the coordinated movement across plasma and vacuolar membranes of ions (e.g. K^+, Cl^-) and molecules (e.g. malate, glucose), which by determining the osmotically driven movement of water, affect cells turgor (Pandey et al., 2007; Wang et al., 2014; Demes et al., 2020; Flütsch et al., 2020a, 2020b; Cubero-Font and De Angeli, 2021). The passage of ions across membranes is dependent upon the activity of different ion channels, the gating of which depends on several actors (e.g. membrane potential, phosphorylation, cytosolic pH, cytosolic Ca^{2+}; reviewed in Jezek and Blatt, 2017). In response to drought, plants synthesize the hormone abscisic acid (ABA) that induces stomatal closure through both Ca^{2+}-dependent and Ca^{2+}-independent signaling pathways (MacRobbie, 1992; Laanemets et al., 2013; Huang et al., 2019). Guard cells were among the first cell types where in vivo analyses of cytosolic Ca^{2+} dynamics were studied by using firstly, Ca^{2+} sensitive dyes and later, genetically encoded Ca^{2+} sensors (McAinsh et al., 1995; Allen et al., 1999; Garcia-Mata et al., 2003). Years of intense studies have shown that ABA, PM hyperpolarization, ROS, external Ca^{2+}, among other stimuli, activate PM Ca^{2+} permeable channels leading to cytosolic Ca^{2+} increases (Hamilton et al., 2000; Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003). Interestingly, in guard cells, cytosolic Ca^{2+} increases often occur in the form of repetitive Ca^{2+} oscillations whose frequency, duration, amplitude, and transient number, which are linked to fluctuations in the membrane voltage and in ions' fluxes, regulate the stomatal aperture (Allen et al., 2001; Minguet-Parramona et al., 2016). Experimental evidence demonstrated that in several cases the generation of cytosolic Ca^{2+} transients and oscillations were dependent upon both an influx of Ca^{2+} from the apoplast (Hamilton et al., 2000; Pei et al., 2000; Murata et al., 2001), and its release from internal stores (Garcia-Mata et al., 2003). Molecules like nitric oxide (NO), cyclic ADP-ribose (cADPR), IP3, and inositol hexakisphosphate (IP6) were shown to activate endomembrane channels, thus promoting Ca^{2+} release from internal stores (Alexandre et al., 1990; Muir and Sanders, 1996; Wu et al., 1997; Leckie et al., 1998; Grabov and Blatt, 1999; Garcia-Mata et al., 2003; Lemtiri-Chlieh et al., 2003; reviewed in Jezek and Blatt, 2017). Of note, ABA activates the Ca^{2+} permeable channels at the PM (Hamilton et al., 2000; Pei et al., 2000, Murata et al., 2001; Kwak et al., 2003) and the consequent influx of Ca^{2+} into the cytosol stimulates the Ca^{2+} release from intracellular stores, a process described as Ca^{2+}-induced Ca^{2+} release (CICR; Grabov and Blatt, 1998, 1999). While computational and modeling approaches have indicated that the release of Ca^{2+} from internal stores may account for more than 95% of the Ca^{2+} entering the cytosol during Ca^{2+} transients increase (Chen et al., 2012; Wang et al., 2014; Minguet-Parramona et al., 2016), the molecular identity of channels or transporters mediating this Ca^{2+} release lags behind.

Since the slow vacuolar ion channel coded by the At*TPC1* gene (Peiter et al., 2005) is permeable to both K^+ and Ca^{2+} and its activity is modulated by cytosolic Ca^{2+}, AtTPC1 was one of the main candidates predicted to be involved in the CICR process (Hedrich and Neher, 1987; Ward and Schroeder, 1994; Peiter et al., 2005; Gradogna et al., 2009; Dindas et al., 2021). Although data showed that AtTPC1 was required for the inhibition of the light-induced stomatal opening in the presence of external ABA (Peiter et al., 2005), it was later demonstrated that the *tpc1* null mutant closes stomata like the wild-type in response to ABA, and Methyl Jasmonate (MeJA), but not in response to external Ca^{2+} (Islam et al., 2010). Interestingly, when cytosolic Ca^{2+} was monitored in Arabidopsis guard cells expressing the Cameleon Ca^{2+} sensor (Box 1, Figure 2), *tpc1* did not show any difference in the Ca^{2+}, ABA, and MeJA induced cytosolic Ca^{2+} oscillations compared to the wild-type (Islam et al., 2010). Thus, apparently, in guard cells a direct contribution of AtTPC1 in the CICR process is negligible.

On the other hand, the importance of the transport of ions across the tonoplast for the proper regulation of cytosolic Ca^{2+} dynamics has been demonstrated. The

Arabidopsis De-Etiolated 3 mutant (*det3*) lacking the subunit C of the vacuolar H^+-ATPase showed, in guard cells, altered cytosolic Ca^{2+} dynamics in response to external Ca^{2+} and ROS (i.e. H_2O_2) compared to wild-type (Allen et al., 2000). To be precise, whereas in the wild-type external Ca^{2+} and H_2O_2 induced characteristic cytosolic Ca^{2+} oscillations, the *det3* mutant showed sustained cytosolic Ca^{2+} increases and did not close the stomata (Allen et al., 2000). Conversely, in the same mutant, ABA was effective in inducing stomatal closure, showing a wild-type pattern of cytosolic Ca^{2+} oscillations (Allen et al., 2000). Thus, it is plausible that the *det3* mutant, which is defective in H^+ transport across the tonoplast, also presents an impaired Ca^{2+} uptake in the subcellular compartments such as the vacuole and possibly the ER (Schumacher et al., 1999; Allen et al., 2000). The demonstration that several Ca^{2+} transport systems, including CAXs and Ca^{2+}-ATPases (Box 2) catalyze a Ca^{2+}/H^+ exchange (Bonza and De Michelis, 2011; Demidchik et al., 2018) supports the prediction that defects in H^+ transport can also affect Ca^{2+} transport and its homeostasis (Hills et al., 2012; Wang et al., 2014; Jezek and Blatt, 2017; Dindas et al., 2021). At present, to the best of our knowledge, no attempts to investigate vacuolar and ER Ca^{2+} dynamics during stomatal movements have been made. As a consequence, the analysis of Ca^{2+} dynamics in these two compartments is an important challenge for future research. Available mutants like the Arabidopsis *aca4/aca11*, and *aca1/aca2/aca7* (Hilleary et al., 2020; Ishka et al., 2021) already represent an important genetic resource to perform analyses in this direction. In support of this, analyses of stomatal conductance carried out in these two mutants showed kinetics that slowed significantly on repeated CO_2 elevations compared to the wild-type. These results underpin an important role played by the pools of endomembrane Ca^{2+} in a sort of a "carbon memory" of stomatal responsiveness to light and CO_2 that ultimately affects photosynthesis and water use by the plant (Jezek et al., 2021). Besides ER and vacuole, analyses of nuclear, mitochondrial, peroxisomal, and chloroplasts Ca^{2+} dynamics have been carried out in guard cells, but their contribution to the regulation of stomatal movements is still missing. Stimuli, such as hyperosmotic and hypoosmotic stress also induce, besides a $[Ca^{2+}]_{cyt}$ increase, nuclear and mitochondrial $[Ca^{2+}]$ rises (Loro et al., 2012) and PM hyperpolarization triggers cytosolic and peroxisomal Ca^{2+} increases (Costa et al., 2010). Of note, it was demonstrated that the Arabidopsis *cmcu*-KO mutant failed to close the stomata in response to the osmotic stress and lost water faster compared to the wild-type under drought stress (Teardo et al., 2019). Unfortunately, analyses of cytosolic and chloroplasts Ca^{2+} dynamics in *cmcu*-KO guard cells are lacking. On the other hand, the dual localization of cMCU to both chloroplasts and mitochondria would also require the analysis of mitochondrial Ca^{2+} dynamics. As an intriguing component of the chloroplast Ca^{2+} regulatory network, the Ca^{2+} sensing protein CAS localized in chloroplast thylakoid membranes has been associated with guard cell dynamics. In particular, the lack of AtCAS activity in Arabidopsis, prevents the cytosolic Ca^{2+} increase in guard cells and the stomatal closure induced by the administration of external Ca^{2+} (Nomura et al., 2008; Weinl et al., 2008). Nevertheless, the channels and/or Ca^{2+} transporters involved in this mechanism are unknown.

The large size of guard cells' chloroplasts and the fact that their imaging is relatively easy, allowed Ca^{2+} imaging analyses in single organelles (Loro et al., 2016). This analysis showed that the transition from high-to-low-intensity blue light (needed to excite the Cameleon Ca^{2+} sensor; Box 1, Figure 2), induces a transient Ca^{2+} increase in the stroma. Interestingly, the stromal $[Ca^{2+}]$ rise was characterized by two components: (1) a series of Ca^{2+} spiking superimposed to (2) a gradual sustained Ca^{2+} increase (Loro et al., 2016). These Ca^{2+} spikes were dependent on the availability of cytosolic Ca^{2+} and were not synchronized between individual chloroplasts of the same cell. In contrast, the gradual sustained Ca^{2+} increase occurred independently of cytosolic Ca^{2+}, suggesting an intraorganellar Ca^{2+} release (Loro et al., 2016). At present, the molecular identity of the channels and/or transporters involved both in the generation of the sustained and transient stromal Ca^{2+} increases are not known, but the Arabidopsis AtBICATs, and AtcMCU channels are obvious candidates (Figure 3; Frank et al., 2019, Teardo et al., 2019).

In the future, the combination of modeling, in vivo imaging, and genetics will be instrumental to better define the role played by the subcellular compartments in the stomatal movements.

Contribution of organellar/cytosolic calcium homeostasis in plant developmental processes

As reported above organellar Ca^{2+} dynamics have been mainly studied in relation to plant responses to both abiotic and biotic stresses. Less is known about their functions during developmental processes, and only a few cases have reported on the roles of different subcellular compartments in the regulation of cytosolic Ca^{2+} dynamics during development. Nevertheless, plants have at least two well-known systems where a precise spatiotemporal regulation of $[Ca^{2+}]_{cyt}$ is required for the execution of the proper growth processes: pollen tubes (PTs) and root hairs (RHs; Schoenaers et al., 2017). While PTs provide for water-independent propagation of species, RHs confer an increase in root surface area which enhances nutrient and water uptake and better anchor plants to the soil. PTs and RHs are filamentous cells that grow unidirectionally by depositing flexible cell wall material at one side of the cell and exploiting turgor pressure as the driving force for elongation, a process also referred to as tip growth (Schiefelbein et al., 1993; Feijó et al., 2004). Although these tip-growing systems are very similar, they

also display differences in their growth-related molecular machineries (Feijó et al., 2004).

Role of organellar calcium in directing pollen tube tip growth

Flowering plant reproduction comprises several sequential steps from pollination to fertilization. PTs growth is essential for reproduction, and they have long been considered outstanding models for cell biology for a variety of reasons. One important feature of PTs is their prominent dependence on ion dynamics to promote and regulate growth (Michard et al., 2017). PTs appropriately adjust turgor pressure by adapting to changes in external osmolarity (Hill et al., 2012). In 1963, it was revealed that Ca^{2+} is essential for in vitro PTs cultures (Brewbaker and Kwack, 1963), and the relationship between $[Ca^{2+}]$ and PTs growth has been extensively examined. By using both Ca^{2+} sensitive dyes or genetically encoded Ca^{2+} sensors, it was evident that a tip-focused $[Ca^{2+}]_{cyt}$ gradient occurred in growing PTs showing regular oscillations that correlate with growth (Holdaway-Clarke et al., 1997; Iwano et al., 2009; Michard et al., 2011). It was also shown that the perturbation of the tip $[Ca^{2+}]$ gradient may affect the growth as well as the viability of PTs (Feijó et al., 2001; Zhang et al., 2007; Winship et al., 2017). As in other cell systems, there is clear evidence that the dynamics of Ca^{2+} signals in PTs are largely controlled by Ca^{2+} influx and Ca^{2+} efflux through the PM (Box 2). Just to cite some examples, GLRs (Michard et al., 2011; Wudick et al., 2018), CNGCs (Frietsch et al., 2007; Gao et al., 2016; Pan et al., 2019), and isoforms of PIIB-Ca^{2+}-ATPase (Schiøtt et al., 2004; Ishka et al., 2021) were demonstrated to be required for the proper PT elongation and growth. In a few cases, it was also demonstrated that growth defects were linked to deregulated tip $[Ca^{2+}]$ oscillations (Michard et al., 2011, Chen et al., 2015).

Knowledge about the role of subcellular compartments in the regulation of the tip-Ca^{2+} dynamics is rather limited, but some research has highlighted a potential role of ER and mitochondrial Ca^{2+} in this process (e.g. Holdaway-Clarke et al., 1997; Colaço et al., 2012; Ishka et al., 2021). Of interest, was the demonstration that the treatment of PTs with caffeine reversibly stops their growth and dissipates the tip-Ca^{2+} gradient (Pierson et al., 1996; Diao et al., 2018). In mammalian cells, caffeine is known to promote the release of Ca^{2+} from the ER through the activation of IP3 receptors (IP3Rs; Taylor and Tovey, 2010; Foskett et al., 2007). Thus, it is reasonable to hypothesize that its effect on pollen could depend on a disturbance of cytosolic Ca^{2+}, dependent on a Ca^{2+} release from the ER. However, no IP3Rs have been identified in superior plants (Edel et al., 2017), and no caffeine-dependent ER-Ca^{2+} release has been reported so far. Nonetheless, the role of Ca^{2+} transport across the ER membrane in the regulation of PTs growth was first highlighted by Iwano et al. (2009) who analyzed Ca^{2+} dynamics in the cytosol and ER lumen of Arabidopsis PTs by using Cameleon Ca^{2+} sensors localized in the two compartments (YC4.6 and YC3.6 in the ER and in the cytosol, respectively; Iwano et al., 2009). The administration of cyclopiazonic acid that inhibits the activity of ER-localised PIIA-type Ca^{2+}-ATPases triggered the PTs' growth arrest and an ER luminal $[Ca^{2+}]$ decrease (Iwano et al., 2009). This result allowed the authors to ascribe a role of ER in the fine regulation of the tip-focused $[Ca^{2+}]_{cyt}$ gradient (Iwano et al., 2009). To go deeper inside the ER/cytosolic Ca^{2+} relationships during PTs growth, simultaneous observations of Ca^{2+} dynamics in these two compartments would be required. Whereas there is no genetic evidence that the lack of the ER PIIA-type Ca^{2+}-ATPase AtECA1 activity compromises pollen development and plant fertility, a very recent work showed that the ablation of three ER-localized PIIB-type Ca^{2+}-ATPases, AtACA1, 2, and 7 strongly compromised pollen fertility, with a decrease in pollen transmission efficiency (Ishka et al., 2021). Single knockout mutants did not show any phenotype demonstrating the existence of functional redundancy (Ishka et al., 2021). Neither cytosolic nor ER Ca^{2+} dynamics were however analyzed.

As reported in the previous sections, plant mitochondria accumulate Ca^{2+} possibly through the activity of the MCU complex (MCUC; Wagner et al., 2015; Teardo et al., 2017), but a demonstration that *in planta* this complex represents the main route for Ca^{2+} import is still missing. Nevertheless, in Arabidopsis, there are six MCU isoforms (Stael et al., 2012) and experimental evidence supports a role for AtMCU1 and AtMCU2 in the regulation of pollen germination. Selles et al. (2018) showed that AtMCU1 and AtMCU2 are expressed in the vegetative cell and in the germinated PTs but were not detectable in the embryo sac. Remarkably, pollen grains germination and PTs elongation were reduced in vitro in *mcu2*-KO mutants and this ultimately led to the limited paternal transmission of *mcu2*-KO to the progeny. No reproductive phenotype could be detected in *mcu1*-KO mutants. These results tend to suggest that mitochondrial Ca^{2+} homeostasis is an additional player in pollen germination and PTs growth. However, no measurements of both cytosolic and mitochondrial Ca^{2+} were carried out in the PTs of these mutant lines, thus the conclusion that the observed phenotype is directly linked to a deregulated Ca^{2+} transport could not be drawn yet. Similar to what was suggested for the ER, simultaneous analysis of Ca^{2+} dynamics during PTs growth in the two compartments would be desirable.

Another link in support of a role played by the Ca^{2+} handling by subcellular compartments in the regulation of PT growth came from the indirect evidence that tonoplast-localized Ca^{2+} sensors are required to control the developmental process. In Arabidopsis, the calcineurin B-like proteins AtCBL2 and AtCBL3 are significantly expressed in pollen and PTs. AtCBL2 and AtCBL3 were described as interacting with the AtCIPK12 at the tonoplast, with evident phenotypic consequences at the level of vacuolar morphology and PTs' polar growth upon mutation or overexpression of *CBL2/3* or *CIPK12* (Steinhorst et al., 2015). Interestingly, Tang et al. (2020) demonstrated that seedlings of the Arabidopsis *cbl2/cbl3* mutant were

severely stunted under low K^+ conditions, a phenotype due to an impairment in K^+ remobilization from vacuoles. Therefore, it is tempting to speculate that a similar mechanism could be present in PTs. Because AtCBL2 and AtCBL3 are attached to the tonoplast facing the cytosolic side, it is conceivable that they are sensitive to changes in the cytosolic Ca^{2+} in the tonoplast microdomain (Knight et al., 1996). At present, there is no evidence that the Ca^{2+} regulation of the CBL2/3-CIPK12 complex in the tonoplast is released from the vacuole, but this is a suggested hypothesis that may require further research.

Role of organellar calcium in directing root hair tip growth

RHs are tubular extensions from the surface of root epidermal cells (Schoenaers et al., 2017). This "hairlike" morphology is essential for its role in nutrient and water uptake, plant anchoring in the soil, and microbial interactions (Véry and Davies, 2000; Charpentier and Oldroyd, 2013). In RHs, the tip $[Ca^{2+}]$ gradient (Wymer et al., 1997; Monshausen et al,. 2007; 2008; Candeo et al., 2017) is maintained by an influx of extracellular Ca^{2+} through hyperpolarization-activated plasma membrane-localized Ca^{2+} channels (Véry and Davies, 2000), and CNGCs (Brost et al., 2019). Indeed, similarly to PTs, $[Ca^{2+}]_{cyt}$ has emerged as a key component controlling the RHs growth and its perturbation alters the RHs' proper developmental program (Monshausen et al., 2008). It is conceivable that as in PTs, also in RHs the organellar Ca^{2+} transport can impinge on the cytosolic Ca^{2+} dynamics. However, at present, the information available regarding their possible contribution is very scarce. Attempting a comparison with PTs, we might predict that in RHs, the ER and mitochondria Ca^{2+} transport could also affect the developmental process. Only a few pieces of evidence support a possible role of mitochondria in the RHs development. Wang et al. (2010) demonstrated that treatments of Arabidopsis RHs with latrunculin B or jasplakinolide, which depolymerize and polymerize actin filaments respectively, decreased the mitochondrial inner membrane potential, triggered a release of Ca^{2+} from the mitochondria, and induced a $[Ca^{2+}]_{cyt}$ increase in the RHs (Wang et al., 2010). Moreover, the authors highlighted the presence of a $[Ca^{2+}]$ gradient in mitochondria from the tip to the base of the RH that was disrupted by the drugs. Even if it is suggestive of a role of mitochondria in the regulation of $[Ca^{2+}]_{cyt}$ in RHs development, genetic evidence in support of this hypothesis is lacking. Teardo et al. (2017) analyzed the roots of seedlings of the *mcu1*-KO and At*MCU1* overexpressing plants without reporting any abnormalities in the RHs development. However, Arabidopsis has six MCU isoforms and at least two of them (At*MCU1* and Atc*MCU*) are expressed in RHs (source: eFP browser; Winter et al., 2007) and all are expressed in roots (source: Geneinvestigator; Hruz et al., 2008; Klepikova et al., 2016), therefore the absence of clear RHs phenotypes might be due to functional redundancy. The generation of higher order *mcu* mutants should be pursued to analyze their involvement in mitochondrial Ca^{2+} regulation during RHs development.

Role of calcium in root tip growth

Primary root tip growth is another plant development program that seems to be subject to the regulation of $[Ca^{2+}]_{cyt}$ dynamics by subcellular compartments. Leitão et al. (2019) showed that in Arabidopsis, nuclear Ca^{2+} signaling is elemental for proper primary root development, including meristem development and auxin homeostasis. The genetic ablation of the NE localized AtDMI1, a functional analog of MtDMI1 in root symbioses thus a putative ion channel permeable to K^+ that counterbalances the charge caused by the influx of Ca^{2+} into the nucleus (Granqvist et al., 2012), was able to modulate the nuclear Ca^{2+} signatures, and primary root development (Leitão et al., 2019; Figure 3). However, the molecular identity of the Ca^{2+} permeable channel responsible for the generation of the nuclear Ca^{2+} transient is still unknown. Based on structural homology analyses, AtDMI1 can be classified as a BK channel (Kim et al., 2019). Interestingly, the LjCASTOR BK channel of *Lotus japonicus*, when expressed in HEK293 cells as a truncated form, lacking the N-terminus and two transmembrane domains, was shown to be both permeable to and regulated by Ca^{2+} (Kim et al., 2019). Based on this piece of evidence we might speculate that AtDMI1 could also be permeable to Ca^{2+} but this needs to be experimentally demonstrated. Nevertheless, it would be of major interest to directly monitor the Ca^{2+} in the NE to have direct proof that this compartment, in continuity with the ER, is responsible for the observed nucleoplasm Ca^{2+} transient.

Conclusions

Since the publication of the seminal review from McAinsh and Pittman (2009) in which the authors provided and discussed the first available evidence about the role of subcellular compartments in the regulation of the cellular Ca^{2+} homeostasis in plant cells, much progress has been made. In this Update, we have recapitulated the experimental proofs collected in recent years supporting the role of the intracellular compartments in the generation and shaping of $[Ca^{2+}]_{cyt}$ increases observed in response to environmental and developmental stimuli (Figure 4). Forward and reverse genetic approaches coupled to the use of imaging tools have now shed light on key roles played by vacuole, chloroplast/plastid, and ER/NE in the shaping of cytosolic Ca^{2+} dynamics in different physiological/stressful conditions. In some cases, channels and transporters directly involved in the movement of Ca^{2+} across the membranes of these compartments were also identified (Box 2; Figure 3). However, despite this increased knowledge, understanding of the role played by mitochondria in the regulation of cytosolic Ca^{2+} dynamics is still limited. Equally, the organelles' contribution in Ca^{2+}-dependent stomatal movements needs experimental confirmation (see "Outstanding questions").

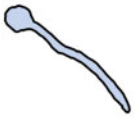
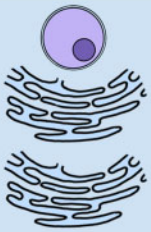
Tissue or cell types	Type of stimulus	Protein name	Compartment involved	References
	Drought stress, CO_2	AtTPC1 AtcMCU AtACA4 AtACA11 AtACA1 AtACA2 AtACA7		Peitier et al., 2005 Islam et al., 2010 Teardo et al., 2019 Jezek et al., 2021 Jezek et al., 2021
	Plant-pathogen interaction; wounding, high light	AtACA4 AtACA11 NbCA1 AtACA1 AtACA2 AtACA7 AtTPC1		Hilleary et al., 2020 Zhu et al., 2010 Ishka et al., 2021 Kiep et al., 2015; Vincent et al., 2017
	Pollen germination and development	AtECAs AtACA1 AtACA2 AtACA7		Iwano et al., 2009 Ishka et al., 2021
	Root hairs growth	???		Wang et al., 2010
	Salt and osmotic stress, hydrotropism; root meristem development	AtDMI1 AtECA1 AtCCX2		Leitao et al. 2019 Shkolnik et al. 2018 Corso et al. 2018

Figure 4 Summary of the Ca^{2+} transport mechanisms localized in the subcellular compartments of plant cells involved in the regulation of cytosolic Ca^{2+} dynamics for which a physiological response in different plant tissues/cell types was demonstrated.

Much work needs to be done. Forward genetic screening based on the use of recently developed organelle localized Ca^{2+} sensors (Box 1; Figure 2), could provide a valuable strategy for the identification of additional players involved in the accumulation and release of Ca^{2+} from internal stores. Moreover, besides using model plants, the identification and the definition of the physiological role of intracellularly localized Ca^{2+} permeable channels and transporters should be extended to important crop species such as rice, for which preliminary results are already available (Singh et al., 2014).

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OUTSTANDING QUESTIONS

- How are the Ca^{2+} transport systems of different subcellular compartments regulated?
- Does mitochondrial Ca^{2+} transport impinge on cytosolic dynamics in the responses to stress or in developmental processes?
- What is the role of AtcMCU in mitochondria?
- What is the role of GA in Ca^{2+} signaling?
- What is the role of AtECA3 and OsACA7 in the Golgi apparatus?
- Which are the organellar Ca^{2+} transport systems involved in the regulation of stomatal movements?
- Can the design of new genetic screenings help to identify new Ca^{2+} transporters of subcellular compartments?

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