The contribution of organelles to plant intracellular Calcium signalling

Alex Costa[1,2*], Lorella Navazio[3,4*], Ildiko Szabo[3,4,5]

1Department of Biosciences, University of Milan, Via G. Celoria 26, 20133 Milan, Italy

2 Institute of Biophysics, Consiglio Nazionale delle Ricerche, Via G. Celoria 26, 20133 Milan, Italy

3 Department of Biology, University of Padova, Via U. Bassi 58/B, 35131 Padova, Italy

4 Botanical Garden, University of Padova, Via Orto Botanico 15, 35123 Padova, Italy

5 Institute of Neurosciences, Consiglio Nazionale delle Ricerche, Via U. Bassi 58/B, 35131, Padova, Italy

Correspondence should be sent to: ildiko.szabo@unipd.it

*Equal contribution

Highlights

The present review summarizes our current knowledge regarding organellar Ca^{2+} signaling and its consequences on plant physiology.

Abstract

Calcium (Ca^{2+}) is among the most important intracellular messengers in living organisms. Understanding of the players and dynamics of Ca^{2+} signalling pathways in plants may help to unravel the molecular basis of their exceptional flexibility to respond and to adapt to different stimuli. In the present review we focus on new tools that have recently revolutionized our view of organellar Ca^{2+} signalling as well as on the current knowledge regarding the pathways mediating Ca^{2+} fluxes across intracellular membranes. The contribution of organelles and cellular subcompartments to the orchestrated response via Ca^{2+} signalling within a cell is also discussed, underlining the fact that one of the greatest challenges in the field is the elucidation of how influx and efflux Ca^{2+} transporters/channels are regulated in a concerted manner to translate specific information into a Ca^{2+} signature.

Introduction

Changes in Ca^{2+} levels within plant cells can be considered as hallmarks of a plethora of processes as growth, differentiation, regulation of stomata opening, induction of pathogen defense responses, establishment of plant-microbe symbioses and stress adaptation. Indeed, each of these processes are associated with specific "Ca^{2+} signatures", arising from variations of Ca^{2+} concentration characterized by a unique amplitude, frequency and duration within the cytosol and, in some cases, in a given intracellular compartment (see e.g. Evans *et al.*, 2001; Monshausen, 2012; Trewavas *et al.*, 1996; Whalley and Knight, 2013; Xiong *et al.*, 2006). Thus, the concentration of free Ca^{2+} in the cytosol (Ca^{2+}_{cyt}) is crucial for Ca^{2+}-based signalling. Tight regulation of the $[Ca^{2+}]_{cyt}$ is mandatory because above 10^{-4} M sustained increases can lead to protein and nucleic acid aggregation and to precipitation of phosphates, thus causing damages to membranes and organelles, ultimately leading to a generalized cytotoxicity. On the other hand, rapid and transient increase of cytosolic Ca^{2+} concentration via Ca^{2+} channels mediating either Ca^{2+} influx of the ion from the extracellular milieu or the temporary release of the ion from intracellular stores, have enabled Ca^{2+} to function as a versatile second messenger in basically all physiological systems (Dodd *et al.*, 2010).

Upon stimulation, $[Ca^{2+}]_{cyt}$ increases from approximately 10^{-7} M to 10^{-6} M, relaying an external stimulus to the intracellular milieu and allowing to trigger specific biological responses. A set of proteins, that undergo Ca^{2+} binding-induced conformational changes help the cells to decode the signal by responding to the stimulus-induced increases in $[Ca^{2+}]_{cyt}$ (McAinsh and Pittman, 2009). Intracellular organelles may contribute to the regulation of free Ca^{2+} homeostasis in the cytosol, since a fast response of Ca^{2+} levels to environmental cues is ensured by compartmentalization of this cation within the plant cell (Nomura and Shiina, 2014; Stael *et al.*, 2012). In fact, in parallel with the actions of Ca^{2+} influx and Ca^{2+} efflux systems across the plasma membrane (PM), Ca^{2+} sequestration into and release from the intracellular compartments are equally important to maintain the transient nature of Ca^{2+} signals (Kudla *et al.*, 2010; Trewavas *et al.*, 1996). Ca^{2+} can be mobilized from storage compartments such as the cell wall/apoplast, vacuole and the endoplasmic reticulum (ER), whereas the nucleus, as well as chloroplasts and mitochondria can generate also intra-organellar Ca^{2+} signals (Stael *et al*., 2012). Changes in free $[Ca^{2+}]$ in a given organelle in turn may influence its function.

Ca^{2+}-based signalling systems have long been described as over-simplified linear pathways (with a stimulus generating transient $[Ca^{2+}]_{cyt}$ elevation that in turn leads to a specific response). Since plants can be challenged by several stimuli at the same time – most of which involve changes in $[Ca^{2+}]_{cyt}$ - the final response often implies a complex network of intersecting signal transduction pathways, each specific for a given stimulus. Thus, it is becoming increasingly evident that Ca^{2+} signalling systems are intrinsically complex networks comprising many interconnected nodes and hubs (Dodd *et al*., 2010).

In the following sections an overview of the recently developed toolkit to measure time-resolved organellar Ca^{2+} signalling in intact plants as well as plant cell suspension cultures is provided, along with discussion of the possible Ca^{2+}-permeable channels in the various organelles. We will give special emphasis to bioenergetic organelles mitochondria and chloroplasts as well as to peroxisomes and ER, whereas we advise the readers to consult the review on nuclear Ca^{2+} signalling that is published in the present special issue for information on the participation of the nucleus to the Ca^{2+} signalling network (Charpentier *et al*., 2018, this issue).

General molecular players of plant Ca^{2+} signalling

In plants, shaping of the Ca^{2+} signature with defined spatial and temporal characteristics and specificity in Ca^{2+}-based signalling is achieved through the interplay of Ca^{2+} signatures together with Ca^{2+}-binding proteins that act to decode or interpret Ca^{2+} level increases (e.g. Tang and Luan, 2017).

Ca^{2+} signatures are decoded by Ca^{2+} binding sensor proteins that act either as primary responders or signal relays (DeFalco *et al.*, 2009; Tang and Luan, 2017; Zhu, 2016). Ca^{2+}-dependent protein kinases (CPDK) are primary responders, while calmodulins (CaMs), calmodulin-like proteins (CMLs) and calcineurin B-like proteins (CBLs) are part of the latter group. These Ca^{2+} sensors trigger a downstream signalling cascade that culminates in changes in gene and protein expression, metabolic activity and developmental changes (see e.g. Lenzoni *et al.*, 2017). Excellent, recent reviews underline the crucial role of the above protein families in global Ca^{2+} signalling (Kudla *et al.*, 2018; Ranty *et al.*, 2016; Simeunovic *et al.*, 2016; Tang and Luan, 2017), therefore the present review mentions only briefly their contribution to Ca^{2+}signalling.

The other crucial proteins for Ca^{2+} signalling are those involved in the transport of this ion across biological membranes, namely transporters (active or passive) and channels, that mediate flux of ions against or down the electrochemical gradient, respectively. These proteins include Ca^{2+}-ATPases, cation/proton exchangers (CAXs) and cation/Ca2+ exchangers (CCXs) that are emerging players in an increasing range of cellular and physiological functions (Bose *et al.*, 2011; Corso *et al.*, 2018; Costa *et al.*, 2017; Frei dit Frey *et al.*, 2012; Pittman and Hirschi, 2016). Ca^{2+} permeable channels include members of the glutamate-like receptor family (Forde and Roberts, 2014; Steinhorst and Kudla, 2014; Swarbreck *et al.*, 2013), of cyclic nucleotide-gated channels (CNGCs) (DeFalco *et al.*, 2016; Dietrich *et al.*, 2010) and of mechanosensitive (MSC) channels (Hamilton *et al.*, 2015). Unconventional Ca^{2+} transporting annexin1 is also a possible player (Davies, 2014). In addition, organelle-specific channels such as the vacuolar two-pore cation (TPC) channel (Choi *et al.*, 2017; Choi *et al.*, 2014; Hedrich *et al.*, 2018; Kiep *et al.*, 2015; Peiter *et al.*, 2005; Vincent *et al.*, 2017a) and the mitochondrial calcium uniporter (Teardo *et al.*, 2017; Wagner *et al.*, 2015a; Wagner *et al.*, 2016) contribute to shaping Ca^{2+} signalling.

We will refer to the above-mentioned Ca^{2+}-transporting molecules in the context of specific organellar Ca^{2+} signalling in the following sections.

Overview of toolkits to measure plant organellar Ca^{2+} concentrations *in vivo*

Analysis of Ca^{2+} dynamics in living plants was initially addressed by using Ca^{2+} sensitive dyes (e.g. Fura-2, Fura-2 dextran and Ca^{2+} Green Dextran) loaded in guard cells, pollen tubes and root hairs (Ehrhardt *et al.*, 1996; Holdaway-Clarke *et al.*, 1997; McAinsh *et al.*, 1995). The use of these dyes allowed to make fundamental discoveries, but they present some limitations for their requirement to be loaded or manually injected and also because their use suffers from low throughput, variability and is prone to artifacts. Hence,

we feel comfortable to say that analysis of Ca^{2+} dynamics in living plants was revolutionized by the introduction of the Genetically Encoded Ca^{2+} Indicators (GECI) (Perez Koldenkova and Nagai, 2013) that permitted non-invasive monitoring of free Ca^{2+} levels, enabling real-time, spatially and temporally-resolved imaging of Ca^{2+} levels in different cell types and organisms and even in specific sub-cellular compartments by specific targeting of GECIs to organelles (Costa and Kudla, 2015; Stael *et al.*, 2012). Furthermore, the possibility to calibrate GECIs may allow to obtain information on absolute concentrations for different ions (see e.g. Lanquar *et al.*, 2014 for Zn^{2+} and Waadt *et al.*, 2017 for Ca^{2+}).

The first subcompartmental (cytosolic) GECI, exploitable for *in vivo* measurements, was obtained for aequorin in plants (Knight *et al.*, 1991). A year later, aequorin was specifically expressed in animal mitochondria via fusion with the signal sequence-encoding part of a mitochondria-located protein. This study revealed for the first time that mammalian mitochondria can accumulate high concentrations of Ca^{2+} upon stimulation of the cells with histamine, an agonist of the inositol triphosphate receptor located in the ER (Rizzuto *et al.*, 1992). Following these studies, this methodology became widely accepted as a general tool to measure organellar Ca^{2+} changes in the animal field (Bagur and Hajnoczky, 2017; Brini *et al.*, 1999; Ottolini *et al.*, 2014; Rudolf *et al.*, 2003) and to establish the presence of high Ca^{2+} concentration microdomains that are generated at the ER-mitochondria contact site level (Rizzuto *et al.*, 1993; Rizzuto *et al.*, 1998).

In plants, thus far the two main Ca^{2+} indicators used are aequorin and Cameleon (Costa and Kudla, 2015; Knight and Knight, 1995; Mithofer and Mazars, 2002) and importantly, both display a binding affinity for Ca^{2+} that renders them useful to detect changes in Ca^{2+} concentrations in the physiologically occurring ranges (Palmer and Tsien, 2006). As mentioned above, the first GECIs to be developed were the aequorin-based probes, which allowed 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; Knight *et al.*, 1992; Logan and Knight, 2003; Sai and Johnson, 2002). It has been an extraordinary tool to determine the Ca^{2+} dynamics triggered by different stimuli at the level of cell populations or entire plants, forming the basis of our understanding of the *in vivo* dynamics of free Ca^{2+} in plants. Since aequorin is largely insensitive to variations of pH and Mg^{2+} (Brini, 2008), it can be used as a reliable sensor to monitor [Ca^{2+}] changes even in organelles or subcompartments with acidic pH. Furthermore, its bioluminescent properties, high signal-to-noise ratio and lack of damaging excitation light makes it an excellent tool to measure Ca^{2+} levels in chlorophyll-containing tissues even for long time intervals (Marti *et al.*, 2013). Aequorin-based sensors are available for different plant organelles, such as the vacuole (Knight *et al.*, 1996), the nucleus (van Der Luit *et al.*, 1999), the Golgi apparatus (Ordenes *et al.*, 2012), mitochondria (Logan and Knight, 2003) and plastids/chloroplasts (Johnson *et al.*, 1995; Mehlmer *et al.*, 2012; Sello *et al.*, 2016). Concerning these latter organelles, aequorin chimeras have been targeted to the different chloroplast subcompartments, *i.e.* the stroma (Johnson *et al.*, 1995; Sai and Johnson, 2002), the outer and inner membranes of the envelope (Mehlmer *et al.*, 2012) and the thylakoid lumen and membrane (Sello *et al.*, 2018) (Table 1). Aequorin was also targeted to the apoplastic space (Gao *et al.*, 2004). Moreover, the development of novel Bioluminescence Resonance Energy Transfer (BRET)-based GFP-aequorin reporters, initially designed for Ca^{2+} imaging in animal cells (Baubet *et al.*, 2000; Rogers *et al.*, 2005) has overcome one of the major limitations of aequorin, *i.e.* its low amount of emitted light, thus allowing to visualize Ca^{2+} signals propagating over long distances in intact plants (Xiong *et al.*, 2014).

The application of ratiometric Ca^{2+} reporter proteins that are based on combinations of green fluorescent protein (GFP)-related proteins (Cameleons) has greatly advanced the spatio-temporal resolution and sensitivity of Ca^{2+} signalling studies. Cameleons are Förster Resonance Energy Transfer (FRET)-based indicator proteins, which harbor cyan and yellow fluorescent proteins (CFP and YFP or spectral variants of them) linked together by the Ca^{2+}-binding protein calmodulin (CaM) and the CaM-binding peptide M13 (Costa and Kudla, 2015). Binding of Ca^{2+} to each of the four helix loop helix structures of the EF hand motifs present in CaM (one Ca^{2+} ion/EF hand motif) leads to a conformational change resulting in reduced distance between CFP and YFP and increase in FRET. FRET, and thus the [Ca^{2+}] increases, can be conveniently measured by the increase in the ratio between the emission intensity of YFP and CFP upon CFP excitation (Miyawaki *et al.*, 1997). Since the Ca^{2+} recordings with such ratiometric proteins completely rely on ratio shifts, these measurements are not influenced by the actual cellular expression level of the indicators and can also correct for focus changes. CaM-based sensors are available for different intracellular compartments and even for simultaneous measurement of Ca^{2+} dynamics in different subcellular compartments (Costa and Kudla, 2015; Kelner *et al.*, 2018; Krebs *et al.*, 2012).

Other GFP-based Ca^{2+} biosensors, such as for example, Case12, GCaMP3, GCaMP6 (Liu *et al.*, 2017; Vincent *et al.*, 2017a; Vincent *et al.*, 2017b; Zhu *et al.*, 2014) as well-as the green and red variant of GECO1 (G-Geco1 and R-Geco1), have also been successfully applied to measure real-time *in vivo* changes in the cytosol and nucleus (Keinath *et al.*, 2015; Kelner *et al.*, 2018; Ngo *et al.*, 2014; Waadt *et al.*, 2017). Furthermore, a strategy of novel, dual-FP (fluorescent protein) biosensor with large dynamic ranges based on employment of a single FP-cassette that nests a stable reference FP (large Stokes shift LSSmOrange) within a reporter FP (circularly permuted green FP) has been recently set up (Ast *et al.*, 2017). This strategy has been applied to obtain a novel probe from GCaMP6 (Ast *et al.*, 2017). The R-GECO1 and GCaMP biosensors were found to exhibit a significantly higher signal change compared to Cameleon YC3.6 in response to several stimuli (Keinath *et al.*, 2015; Kleist *et al.*, 2017) and the high fluorescent yield of GCaMPs renders these single-fluorescent protein Ca^{2+} sensors particularly suited to whole tissue imaging, which is often required in studies of plant biotic interactions (Vincent *et al.*, 2017b). In addition, the use of red-shifted sensors opens the way to simultaneously apply distinct Ca^{2+} probes localised to different compartments, for example together with Cameleon or other GFP-based GECIs as recently reported by Kelner and colleagues who monitored the cytosolic and nuclear Ca^{2+} dynamics by simultaneously expressing the CG-Geco1 and NR-Geco1 sensors (Kelner *et al.*, 2018). On the other hand, it has to be noted that single-FP sensors cannot quantify absolute [Ca^{2+}] as simply as FRET-based sensors. However, for example in the case of GCaMP6 it has been experimentally determined that exciting GCaMP6 at 410 nm leads to fluorescence emission, which is not Ca^{2+} dependent. As a consequence, the ratio between 474 nm and 410 nm excitation wavelengths is proportional to [Ca^{2+}] (Patron *et al.*, 2014). In summary, in spite of the wide variety of currently available GECIs, for the moment aequorin-based probes still remain the method of election when accurate quantifications of Ca^{2+} levels are needed (Ottolini *et al.*, 2014).

As mentioned above, organelle-targeted, bioluminescent or fluorescent GECIs, summarized in Figure 1, have greatly advanced the field of organellar Ca^{2+} signalling in both animals and plants. In the following sections results obtained exploiting these various probes in different organelles will be reported and compared. Table 1 summarizes the measured affinities (expressed as K_D values) of different, organelle-targeted GECIs.

The main Ca^{2+} storage compartment: the vacuole

Plant vacuoles are large organelles with a diameter of 20 to 40 µm, occupying 80-90% of the cell volume in mature plant cells. Rather than being just the plant counterparts of animal lysosomes, they actually fulfill many different roles, such as the temporary storage of primary metabolites, or the permanent accumulation of secondary metabolites, including potentially toxic compounds (Kruger and Schumacher, 2017; Shimada *et al.*, 2018). Similarly to animal lysosomes, they store high concentrations of Ca^{2+} and Na^{+} (Peiter, 2011). In the central vacuole the concentration of Ca^{2+} can reach values as high as 50 mM. Nevertheless, most of it is present in bound form (the free vacuolar Ca^{2+} concentration ranges from 0.2 mM to 1-5 mM) (Table 2) (Felle, 1989) and therefore is not readily available for Ca^{2+} signalling (Conn and Gilliham, 2010). However, vacuolar Ca^{2+} might indirectly affect the signalling by influencing the activity of ion transporters localized on the vacuolar membrane (Peiter, 2011). In an early pioneering study, the targeting of an aequorin probe to the cytosolic face of the vacuolar membrane provided evidence for the participation of the vacuole in Ca^{2+} signalling activated by cold (Knight *et al.*, 1996). More recently, a Cameleon-based tonoplast-targeted sensor has also been generated, but besides labelling the tonoplast, it was present also in the cytosol, restricting the actual usefulness of such sensor (Krebs *et al.*, 2012). A Ca^{2+} sensor localized to the vacuolar lumen would be extremely useful for the understanding of those Ca^{2+} signalling events in which a contribution from the internal stores has been hypothesized (see also below). Unfortunately, the low pH and the high Ca^{2+} concentration of the vacuolar lumen makes it difficult to use the currently available GECIs to monitor efficiently Ca^{2+} dynamics inside this organelle. Nevertheless, in mammalian cells, a more acidic pH-resistant Ca^{2+} sensor, GEM-GECO1 (Horikawa, 2015) has been successfully targeted to the lysosomal lumen (Albrecht *et al.*, 2015). making it possible to study *in vivo* lysosomal Ca^{2+} dynamics triggered by histamine treatment despite the acidic lumen pH. However, the probe was still pH sensitive making the analysis and interpretation of the data difficult and requiring a tricky pH calibration. An important breakthrough is probably the recent identification of a new pH resistant GFP (Shinoda *et al.*, 2017) that will probably allow the development of new sensors suitable for acidic compartments.

A plethora of transporters and channels are active in the tonoplast, as discovered by direct patch clamping of this organelle (Martinoia *et al.*, 2012; Xu *et al.*, 2015). Many of these transport systems have been molecularly identified during the last few decades (Martinoia *et al.*, 2012; Neuhaus and Trentmann, 2014). Ca^{2+} is taken up into the vacuole likely by two P-type Ca^{2+} pumps, such as calmodulin-regulated autoinhibited Ca^{2+}-ATPases (ACAs), as well as by Ca^{2+}-proton exchangers (CAXs) (Edel *et al.*, 2017), which exhibit a high sequence homology to their yeast counterparts residing also on the vacuolar membrane (Hirschi *et al.*, 1996). ACA pumps exist in at least 10 isoforms in Arabidopsis (Geisler *et al.*, 2000). Activity of the two vacuolar ACA Ca^{2+} pumps AtACA4 and AtACA11 (Lee *et al.*, 2007) has been linked to the control of a salicylic acid-dependent programmed cell death pathway in plants (Boursiac *et al.*, 2010). Among the six CAX members in Arabidopsis, AtCAX1-4 have been shown to locate to vacuoles (Cheng *et al.*, 2002; Pittman *et al.*, 2005). Knock-out mutants of AtCAX1, that is highly expressed in leaf tissue, exhibited altered plant development, perturbed hormone sensitivities, and altered expression of an auxin-regulated promoter-reporter gene fusion (Cheng *et al.*, 2003), while indole-3-acetic acid (IAA) inhibition of abscisic acid (ABA)-induced stomatal closure was found to be impaired in *cax1*, *cax3*, and *cax1/cax3* mutants (Cho *et al.*, 2012). Vacuolar CAX4, that shows low expression level, plays an important function in root growth under heavy

metal stress conditions (Mei *et al.*, 2009). Interestingly, some of the CAXs transport not only Ca^{2+}, but also heavy metals such as Mn^{2+} and Cd^{2+} (Manohar *et al.*, 2011; Martinoia *et al.*, 2012; Pittman and Hirschi, 2016; Socha and Guerinot, 2014).

The vacuole, together with the cell wall/apoplast, are the major Ca^{2+} store and it is generally assumed that Ca^{2+} released from the vacuole provides in several cases substantial contributions for the activation of signal transduction pathways. This assumption is made based on early experiments showing that inositol 1,4,5-trisphosphate ($InsP_3$) releases Ca^{2+} predominantly from the vacuole (Alexandre and Lassalles, 1990; Allen *et al.*, 1995). However, later experiments indicated that in plants, inositol-hexakisphosphate ($InsP_6$) plays a prominent role with respect to $InsP_3$ in intracellular signal transduction (Lemtiri-Chlieh *et al.*, 2003; Munnik and Nielsen, 2011). Among the channels proposed to release Ca^{2+} from the vacuole, the Ca^{2+}-activated two-pore non-selective, Ca^{2+} and K^+-permeable cation channel TPC1 (e.g. Carpaneto and Gradogna, 2018; Peiter *et al.*, 2005), whose structure has been solved (Guo *et al.*, 2016b) acts as tonoplast channel. Since the physiological concentration of K^+ both in the cytosol and inside the vacuole (about 100 mM) is much higher than the concentration of Ca^{2+} (Table 2), K^+ permeation can be expected to be largely facilitated with respect to Ca^{2+} through the channel. Indeed, the ability of TPC1 to conduct Ca^{2+} has long been debated, but combination of the patch-clamp technique with Ca^{2+} detection by fluorescence finally led to the demonstration that Ca^{2+} is able to permeate through TPC1, even if its concentration (0.5 mM) is much lower than that of K^+ (105 mM) in electrophysiological experiments (Carpaneto and Gradogna, 2018; Gradogna *et al.*, 2009). Plants lacking TPC1 are defective in both abscisic acid-induced repression of germination and in the response of stomata to extracellular Ca^{2+}, demonstrating a critical role of the vacuole Ca^{2+}-release channel in various physiological processes of plants (Peiter *et al.*, 2005). Furthermore, the propagation of salt-stress induced long-distance Ca^{2+} waves as well as wounding/herbivory-triggered Ca^{2+} waves were found to be dependent on TPC1 in Arabidopsis (Choi *et al.*, 2014; Kiep *et al.*, 2015). However, other reports dismiss a prominent role of TPC1 in vacuolar Ca^{2+}-release, either assessed by aequorin or direct patch-clamp analyses. For example, unaltered cytosolic Ca^{2+} signals were recorded in intact plants either lacking or overexpressing TPC1 upon exposure to various biotic and abiotic stimuli (Ranf *et al.*, 2008). At physiological pH and Ca^{2+}-gradients, TPC1 was shown to conduct Ca^{2+} into the vacuole, suggesting thus to dissipate rather than to generate cytosolic Ca^{2+} signals (at least during external Ca^{2+} -induced stomatal closure) (Rienmuller *et al.*, 2010). Likewise, the findings that the *fou2* mutant plants harboring a hyperactive TPC1 channel variant (D454N) show an increased vacuolar Ca^{2+} content (Beyhl *et al.*, 2009) and a decreased resting cytosolic Ca^{2+} level compared to WT, argues against a Ca^{2+}-release function of TPC1. The *fou2* mutant has also a slightly lower resting cytosolic [Ca^{2+}] compared to WT, and cytosolic Ca^{2+} increases after wounding were found to be similar in both plants (Lenglet *et al.*, 2017). On the other hand, a recent work, carried out by using the fluorescent Ca^{2+} biosensor GCaMP3, highlighted a functionally relevant interplay between the plant defense co-receptor Brassinosteroid insensitive-associated kinase1 (BAK1), the PM localized glutamate receptors GLR3.3 and GLR3.6, and TPC1 to mediate cytosolic Ca^{2+} elevations following biotic stress such as aphid attack (Vincent *et al.*, 2017a). Interestingly, another work highlighted the importance of endomembrane cation fluxes in controlling the basal level of wound-inducible defense mediator jasmonate acid, thanks to the use of the *fou2* mutant of TPC1 (Lenglet *et al.*, 2017). Thus, altogether TPC1 is emerging as a possible regulator of cytosolic Ca^{2+} signals, although many questions still remain open (Hedrich *et al.*, 2018). The readers are advised to consult excellent reviews on the state of the art and hot topics in vacuolar transport research, including those

discussing the regulation of vacuolar channels by cytoplasmic/luminal factors (Edel *et al.*, 2017; Francisco and Martinoia, 2018; Hedrich, 2012; Martinoia *et al.*, 2012).

A role for endoplasmic reticulum in plant intracellular Ca^{2+} signalling?

Another main intracellular Ca^{2+} store is the ER. Not much is known about the Ca^{2+} storage properties of the plant ER in contrast to the animal field, where it is well explored (Raffaello *et al.*, 2016; Sammels *et al.*, 2010). In animal cells, the total Ca^{2+} concentration in the ER is supposed to be 2 mM, whereas the free Ca^{2+} concentration ranges between 50 µM and 500 µM (Rizzuto *et al.*, 2009; Stael *et al.*, 2012).

The involvement of the ER in Ca^{2+} homeostasis and signalling in plant cells has long been under-appreciated, possibly overshadowed by the prominent role commonly ascribed to the vacuole, and because of the lack, for long time, of direct measurements of luminal [Ca^{2+}] ($[Ca^{2+}]_{ER}$) and its potential variations during signal transduction. Functional conservation of calreticulin as the major high capacity (15-30 mol of Ca^{2+} per mol of protein), low affinity (K_d = 0.5 mM) Ca^{2+} binding protein in the lumen of plant ER (for reviews see e.g. Jia *et al.*, 2009; Mariani *et al.*, 2003) has provided circumstantial evidence for submillimolar $[Ca^{2+}]_{ER}$. In addition to ER Ca^{2+} storage and modulation of Ca^{2+} homeostasis, calreticulin has been shown to function, together with calnexin, as molecular chaperone for glycoprotein folding and quality control in the ER (Jin *et al.*, 2009). Interestingly, overexpression of calreticulin was found to enhance the survival of plants grown in low Ca^{2+} medium (Persson *et al.*, 2001) and to increase plant salinity tolerance (Xiang *et al.*, 2015). The targeting of a Cameleon probe (YC4.6, with two K_D of 58 nM and 14.4 µM) (Table 1) to the ER of pollen tubes has highlighted a potential involvement of the ER in the fine regulation of the tip-focused $[Ca^{2+}]_{cyt}$ gradient required for pollen tube growth (Iwano *et al.*, 2009).

Arabidopsis contains four P(IIA)-type ATPase genes, AtECA1 to AtECA4, which are expressed in all major organs of Arabidopsis. ECA1 knock-out mutants grew poorly on medium with low Ca^{2+} or high Mn^{2+}, indicating that ECA1-mediated uptake of these divalent cations into the ER is required for plant growth under conditions of Mn^{2+} toxicity or Ca^{2+} deficiency (Wu *et al.*, 2002). The silencing of an ER-localized type IIB Ca^{2+}-ATPase (ECA like) in tobacco has been found to alter intracellular Ca^{2+} signalling and accelerate programmed cell death (PCD) during plant innate immune response, indicating that the Ca^{2+} uptake pathway into the ER functions as regulator of PCD (Zhu *et al.*, 2010). Ca^{2+} release from the ER has also been proposed to play an essential role in sieve tube occlusion via Ca^{2+}-dependent forisome dispersion in legumes in response to burning stimuli (Furch *et al.*, 2009; Tuteja *et al.*, 2010). The recently reported targeting of another Cameleon variant, the CRT-D4ER (with a K_D of 195 µM) (Table 1), allowed the dynamic, *in vivo* monitoring of ER luminal Ca^{2+}, showing that the ER may also work as a capacitor/buffer of cytosolic Ca^{2+} transients (Bonza *et al.*, 2013). In fact, cytosolic Ca^{2+} increases triggered by different stimuli (salt stress, external ATP and glutamate) were followed by Ca^{2+} accumulation into the ER lumen, but not by release. Moreover, dynamically, the ER Ca^{2+} rises followed temporally the cytosolic increases showing a slower rate of accumulation and release (Bonza *et al.*, 2013; Corso *et al.*, 2018). Another clue in favor of the ER role as cytosolic Ca^{2+} capacitor is confirmed by the effect of cyclopiazonic acid (CPA) (an inhibitor of IIB Ca^{2+}-ATPase ECA) which reduced the luminal ER Ca^{2+} concentration and increased the cytosolic one (Bonza *et al.*, 2013;

Zuppini *et al.*, 2004), indicating the ECAs as fundamental players for the ER Ca^{2+} homeostasis. Nonetheless, our recent work has demonstrated that the Arabidopsis CCX2 is localized in the ER where it is directly involved in the control of Ca^{2+} fluxes between the ER and the cytosol, playing a key role in the ability of plants to cope with osmotic stresses (Corso *et al.*, 2018). Concerning Ca^{2+}-permeable channels located at higher plant ER, early biochemical studies have indicated the occurrence of ER Ca^{2+} mobilization pathways activated by voltage (Klusener *et al.*, 1995) and two structurally related molecules, namely the pyridine nucleotides derivatives nicotinic acid adenine dinucleotide phosphate (NAADP) (Navazio *et al.*, 2000) and cyclic ADP-ribose (cADPR) (Navazio *et al.*, 2001). The molecular identity of the above voltage- and ligand-gated Ca^{2+}-permeable channels, however, has not been unraveled yet.

The ER has a unique architecture that facilitates the spatial-temporal segregation of biochemical reactions and the establishment of inter-organellar communication networks. Spatially confined ER-PM microdomains are emerging as highly specialized signalling hubs both in animal systems (see e.g. Demaurex and Guido, 2017; Son *et al.*, 2016) and in plants (Bayer *et al.*, 2017). In addition, the continuity between ER membranes and the outer nuclear membrane suggests a potential role of the ER as Ca^{2+} store participating in the repetitive Ca^{2+} release/uptake from the nucleoplasm and perinuclear cytosol during legume symbioses (Capoen *et al.*, 2011). Cyclic nucleotide-gated channels have recently been demonstrated to mediate these nuclear-associated Ca^{2+} oscillations induced in response to beneficial plant microbes during the nitrogen-fixing symbiosis and arbuscular mycorrhizal symbiosis (Charpentier *et al.*, 2016). Structural and functional interactions have been demonstrated to occur between the ER membranes and stromules, dynamic stroma-filled tubules continuously extending and retracting from plastids (Schattat *et al.*, 2011). The occurrence of specific contact sites through which ER and plastids may exchange not only lipids but also ions such as Ca^{2+}, opens up the possibility of a complex and finely-tuned Ca^{2+} regulation, involving potential ER-plastid cross-talks (Mehrshahi *et al.*, 2013) Instead, the role of ER-mitochondria contact sites in shaping the cytosolic Ca^{2+} signalling is well documented in mammals (e.g. Brini *et al.*, 2017; Rizzuto *et al.*, 2012), but not in plants. We can envision such an intimate liason also in the case of plant cells, although direct proof is missing in this case.

The plant Golgi apparatus: a rather unexplored Ca^{2+} store

The Golgi apparatus in plant cells is made of discrete stacks (formerly indicated as dictyosomes) dispersed throughout the cytoplasm and rapidly moving (several μm/s) along the surface of the ER (Robinson *et al.*, 2015). In addition to essential roles in protein glycosylation and trafficking (Vitale and Galili, 2001), the plant Golgi apparatus serves as factory of polysaccharides (hemicellulose and pectins) for the cell wall matrix, whose architecture is known to be regulated by Ca^{2+} (Mravec *et al.*, 2017). Moreover, the Golgi apparatus is the source for exocytotic vesicles, and it is known that exo- and endocytosis can be modulated by Ca^{2+} (Cucu *et al.*, 2017). In view of the unique structural and functional features of the plant Golgi apparatus with respect to animal cells, we can expect that also Ca^{2+} handling by this compartment may exhibit some specificity in plant cells. Compared to the extensive information about Ca^{2+} handling by the Golgi in mammalian cells (see (Pizzo *et al.*, 2011) for a review), knowledge about Ca^{2+} homeostasis and signalling in the plant Golgi is still scarce. Free Ca^{2+} levels in the Golgi ($[Ca^{2+}]_{Golgi}$) were estimated to be around 0.70 μM (Table 2) (Ordenes *et al.*, 2012), a value which is much lower than $[Ca^{2+}]_{Golgi}$ measured in mammalian cells (ranging from ~250 μM in the cis-Golgi to ~130 μM in the trans-Golgi) (Pizzo *et al.,* 2011). This suggests the existence of Ca^{2+}-buffering systems inside the Golgi, and indeed calreticulin has been

reported to be localized at the plant Golgi, in addition to the ER (Nardi *et al.*, 2006; Navazio *et al.*, 2002). Interestingly, transient increases in Ca^{2+} dynamics were observed in response to several abiotic stimuli, such as cold shock, mechanical stimulation and hyperosmotic stress, whereas the administration of the synthetic auxin analogue 2,4-dichlorophenoxy acetic acid (2,4-D) induced a slow decrease of organellar Ca^{2+} (Ordenes *et al.,* 2012). Concerning Ca^{2+} decoding mechanisms, two calmodulin-like proteins from *A. thaliana*, AtCML4 and AtCML5, were found to be localized in vesicular structures between the Golgi and the endosomal system. Nevertheless, their C-terminal CaM domain was found to be exposed to the cytosolic surface of the vesicles, suggesting that they may sense and decode cytosolic, rather than luminal Ca^{2+} signals (Ruge *et al.*, 2016). The nature of Ca^{2+} transporting proteins still awaits clarification. Among the four P(IIA)-type ATPase genes in Arabidopsis, AtECA3 was proposed to function in the transport of Ca^{2+} and Mn^{2+} ions into the Golgi (Mills *et al.*, 2008).

From the data so far available it is clear that the information on the Ca^{2+} toolkit of the plant endomembrane system awaits further investigation on its precise molecular components and on the specific involvement of the different compartments of the plant secretory pathway as Ca^{2+}-mobilizable stores in Ca^{2+}-mediated signal transduction events.

Chloroplasts as Ca^{2+} signal-shaping components in plant cells

Recent studies have revealed that plant mitochondria and chloroplasts respond to biotic and abiotic stresses with specific Ca^{2+} signals (reviewed by (Kmiecik *et al.*, 2016; McAinsh and Pittman, 2009; Nomura and Shiina, 2014; Rocha and Vothknecht, 2012)). Chloroplasts, that possess a high concentration of Ca^{2+}, serve as important intracellular cytosolic "Ca^{2+} capacitors" in plant cells, and they may also influence the entire cellular Ca^{2+} network by modulating cytosolic Ca^{2+} transients. Thus, they can contribute to shaping cytoplasmic Ca^{2+} signatures (Loro *et al.*, 2016; Nomura *et al.*, 2012; Sello *et al.*, 2016).

The predominant portion of the chloroplastic Ca^{2+} (~15 mM) is bound to the negatively charged thylakoid membranes or to Ca^{2+}-binding proteins, keeping the resting free $[Ca^{2+}]_{stroma}$ as low as 150 nM (Table 2) to avoid the precipitation of phosphates (Hochmal *et al.*, 2015). Importantly, this concentration can be actively regulated: light-dependent depletion of cytosolic Ca^{2+} in the vicinity of chloroplasts has been observed in green algae, suggesting that an active Ca^{2+} uptake machinery is present on the envelope membranes, which is regulated by light/dark transitions and/or photosynthesis (Sai and Johnson, 2002). Specific, high-resolution tools have been exploited to monitor and quantify plastid Ca^{2+} dynamics (Table 1). The bioluminescent Ca^{2+} reporter aequorin was targeted to the chloroplast stroma, highlighting induction of Ca^{2+} influx into the stroma upon light-to-dark transition (Sai and Johnson, 2002) as well as a role for stromal Ca^{2+} signals in the activation of plant innate immunity (Nomura *et al.*, 2012; Stael *et al.*, 2015). Constructs encoding YFP-aequorin chimeras targeted to the outer and inner membrane of the chloroplast envelope, in addition to the stroma, are also available to investigate Ca^{2+} dynamics in these compartments (Mehlmer *et al.*, 2012). We have recently used these plastid-targeted aequorin probes to reveal differential stimulus-specific Ca^{2+} responses of amyloplasts *versus* chloroplasts (Sello *et al.*, 2016), suggesting that Ca^{2+} signalling might have specific roles during plastid development. Interestingly, using chloroplast-targeted Cameleon probe, Ca^{2+} spikes could be detected in a large portion (>80%) of guard cell chloroplasts (Loro *et al.*, 2016). The observed unique spiking pattern for each chloroplast strongly suggests that these Ca^{2+}

signals can be modulated at the level of the single organelle (Loro *et al.*, 2016). The reported observations support the concept that Ca^{2+} plays a key role in integrating internal and external stimuli at the level of individual chloroplasts. Ca^{2+} spikes appeared under chloroplast-autonomous control, even though the source of the Ca^{2+} causing the spike may be the cytosol.

It has been hypothesized that opening of individual Ca^{2+} channels following a stimulus from within the chloroplast itself may allow influx of Ca^{2+} from the cytosol along the negative electrochemical gradient across the chloroplast envelope. For the inner envelope membrane, a value of approximately -110 mV has been reported (Wu *et al.*, 1991). However, the nature of such channel(s) remain elusive (for recent reviews see e.g. Carraretto *et al.*, 2016; Finazzi *et al.*, 2015; Pottosin and Shabala, 2015). Light-dependent uptake of Ca^{2+} into isolated chloroplast is thought to be mediated by a Ruthenium Red-sensitive uniport-type carrier in the envelope membrane and to be linked to photosynthetic electron transport via the membrane potential (Kreimer *et al.*, 1985). Electrophysiological studies suggest the existence of voltage-dependent Ca^{2+} uptake activity (the fast-activating cation channel (FACC)) in the inner envelope membrane of pea chloroplasts (Pottosin *et al.*, 2005). However, the molecular identity of FAAC remains elusive and sensitivity to Ruthenium Red has not been investigated. Presuming that the outer membrane is permeable to Ca^{2+} via porin-like molecules (Carraretto *et al.*, 2016; Szabo and Zoratti, 2014), the most promising inner envelope-located candidates include ion channels that may mediate the negative voltage-driven Ca^{2+} uptake across the inner envelope membrane (Heiber *et al.*, 1995). These channel-forming proteins include the plastid-located glutamate receptors GLR3.4 (Teardo *et al.*, 2011; Teardo *et al.*, 2010) and GLR3.5 (Teardo *et al.*, 2015) the mechanosensitive MSL2/3 channels (Haswell and Meyerowitz, 2006), a Ca^{2+}-ATPase like protein (ACA1) (Huang *et al.*, 1993), as well as HMA1 P-type ATPase (Ferro *et al.*, 2010). The possible role, localization (for ACA1) and specificity of the latter two proteins is however highly debated (Hochmal *et al.*, 2015). The recently identified member of the UPF0016 family, the PHOTOSYNTHESIS AFFECTED MUTANT71 (PAM71) located to the thylakoid membrane was reported to function in manganese transport in higher plants (Schneider *et al.*, 2016). The closest homolog of PAM71, PAM71-HL is located to the chloroplast envelope and is likely to exert the same function (Schneider *et al.*, 2016) as the homologs in cyanobacteria are also linked to manganese homeostasis (Gandini *et al.*, 2017). On the other hand, the thylakoid-located PAM71 was proposed to encode a putative Ca^{2+}/H^{+} antiporter with critical functions in the regulation of photosystem II and in chloroplast Ca^{2+} and pH homeostasis in Arabidopsis (Wang *et al.*, 2016a). The possibility that this protein is able to transport manganese in a Ca2+-dependent way or to transport both cations, will have to be explored in a simplified, reconstituted system. A further candidate for Ca^{2+} transport across chloroplast membranes is represented by one of the six homologs of the Ruthenium Red-sensitive mammalian mitochondrial uniporter (MCU), which displays an ambiguous N-terminal sequence, possibly allowing targeting to both mitochondria and chloroplasts (Stael *et al.*, 2012). However, the localization, channel activity, and the permeability for Ca^{2+} of this putative plastidial member of the AtMCU family has not been described up to now, in contrast to four other mitochondria-located AtMCU homologs (Carraretto *et al.*, 2016; Teardo *et al.*, 2017; Wagner *et al.*, 2015a). At present, it is difficult to understand whether the FAAC channel might correspond to one of the above entities. In addition to Ca^{2+}-permeable channels in chloroplasts, other, regulatory cation fluxes may shape the cytosolic Ca^{2+} signature during stress. Stephan and colleagues (Stephan *et al.*, 2016) brought evidence for involvement of two, envelope-located K^{+}/H^{+} antiporters, namely KEA1 and KEA2 in Ca^{2+}-induced cytoplasmic responses during osmotic stress. In particular, the double *kea1/kea2* mutant showed a reduced cytosolic Ca^{2+} level upon treatment with a hyperosmotic sorbitol solution, suggesting that the function of the two K^{+}/H^{+} antiporters is intimately linked

to Ca^{2+} mobilization pathways at the chloroplast membranes under these conditions. However, the exact mode of action is still unclear.

In addition to the above-mentioned ion channels and transporters, several candidate Ca^{2+} binding proteins and Ca^{2+} sensors have been identified in these organelles and shown to critically contribute to Ca^{2+} homeostasis (Hochmal *et al.*, 2015; Rocha and Vothknecht, 2012; Stael *et al.*, 2012). The impact of impaired organellar Ca^{2+} handling for plant physiology has been convincingly illustrated in the cases of the chloroplast-localized Ca^{2+} sensor protein CAS, for the thylakoid-located Post-Floral-specific gene 1 PPF1 and for the glycosyltransferase QUASIMODO1 (QUA1) (Nomura *et al.*, 2008; Petroutsos *et al.*, 2011; Wang *et al.*, 2003; Zheng *et al.*, 2017). In addition, another Ca^{2+} binding protein, CP12 was shown to play an important role in the regulation of the Calvin-Benson-Bassham cycle (Rocha and Vothknecht, 2013). Studies on the thylakoid-localized Ca^{2+}-sensing receptor CAS showed that chloroplasts modulate intracellular Ca^{2+} signals by controlling external Ca^{2+}-induced cytosolic Ca^{2+} transients during stomatal closure (Nomura *et al.*, 2008; Weinl *et al.*, 2008). Indeed, mutation of the putative chloroplastic Ca^{2+} sensor CAS led to impaired stomatal movement and impaired plant growth, although the detailed molecular mechanism underlying CAS-related effects under various conditions has not been fully elucidated yet (Fu *et al.*, 2013; Wang *et al.*, 2016b; Wang *et al.*, 2012). Pathogen-associated molecular pattern (PAMP) signals evoked specific Ca^{2+} signatures in the stroma in chloroplasts and CAS was involved in stromal Ca^{2+} transients (Nomura *et al.*, 2012). CAS, and thus Ca^{2+} was shown to regulate chloroplast salicylic acid (SA) biosynthesis and plants depleted of CAS failed to induce SA production in response to pathogen infection. Transcriptome analysis demonstrated that CAS allowed chloroplast-mediated transcriptional reprogramming during plant immune responses, as expression of several nuclear defense-related genes was shown to be dependent on CAS. Furthermore, activity of MAPK kinases was shown to be regulated in a CAS-dependent manner, suggesting that chloroplast-modulated Ca^{2+} signalling controls the MAPK pathway for the activation of critical components of the retrograde signalling chain (Guo *et al.*, 2016a; Leister *et al.*, 2017). Thus, it is expected that chloroplasts could play pivotal roles in the Ca^{2+} signalling in plant cells upon different stress stimuli, as indeed indicated by recent results linking the CAS protein to chloroplast-dependent Ca^{2+} signalling under salt and drought stresses as well (Zhao *et al.*, 2015; Zheng *et al.*, 2017). Finally, QUA1 was also recently identified as a regulator of $[Ca^{2+}]_{cyt}$ in response to drought and salt stress (Zheng *et al.*, 2017).

In addition to the chloroplast stroma, Ca^{2+} is required for the function of thylakoid lumen-located proteins such as the oxygen evolving complex, suggesting that changes in free $[Ca^{2+}]$ are likely to occur also in the lumen. Recently, aequorin-based chimeras have been targeted to the thylakoid lumen and the stromal surface of the thylakoid membrane (Sello *et al.*, 2018). The design of these thylakoid-specific Ca^{2+} indicators allowed to measure Ca^{2+} concentrations inside and around thylakoids (Table 2) and to monitor dynamic Ca^{2+} changes in the above sub-chloroplast locations in response to different environmental cues.

The availability of this complex toolkit of chloroplast-targeted Ca^{2+} reporters will pave the way for future studies on chloroplast Ca^{2+} homeostasis and signalling, and rapidly advance our understanding of the still enigmatic integration of these organelles in the plant Ca^{2+} signalling network. In summary, a systematic study linking the possible players of chloroplast Ca^{2+} dynamics to specific plant stress responses using envelope, stroma, thylakoid membrane and thylakoid lumen-targeted Ca^{2+} sensors would be of great importance to further highlight the importance of this organelle in global Ca^{2+} signalling within plant cells. Moreover, a promising field of investigation concerns the analysis of Ca^{2+} handling by non-green plastids in non-photosynthetic tissues and organs, such as the root. Indeed, the cell-type specific cytosolic Ca^{2+}

responses of the root to environmental cues (Kiegle *et al.*, 2000) may entail a differential contribution of root plastids. Moreover, it can be envisaged that root plastids may also play relevant roles in Ca^{2+} signalling during plant interactions with microorganisms of the rhizosphere, either pathogenic or beneficial.

Mitochondrial Ca^{2+} signalling in plants

Plant mitochondrial Ca^{2+} signalling has recently been reviewed (Stael *et al.,* 2012; Nomura and Shiina, 2014; Carraretto *et al.,* 2016; Wagner *et al.*, 2016), therefore here we prevalently focus on the missing links to understand the role(s) played by mitochondria in Ca^{2+} signalling processes. The emerging idea is that, similarly to animal cells, plant mitochondria can play a role in the modulation of cytosolic Ca^{2+} signatures, hence participating in the general intracellular Ca^{2+} homeostasis.

The complex series of redox reactions of the mitochondrial electron transport chain (ETC) coupled to proton movement against the electrochemical gradient across the inner mitochondrial membrane (IMM) generates a proton motive force (pmf) composed of a proton gradient (ΔpH) across the IMM of about 0.9 pH units, and of an electric component (Δψ) reaching values of around -180 mV / -220 mV (Poburko *et al.*, 2011; Szabo and Zoratti, 2014). The generated pmf is exploited to synthetize ATP, and for the import of proteins as well as of several charged substrates and cofactors that are translocated into the matrix via specialized co-transporters (Lee and Millar, 2016). Moreover, the negative matrix-side Δψ drives the import of positively charged ions, like Ca^{2+}, which flux into the matrix passively through channels reaching free Ca^{2+} concentrations with values ranging from 100 to 600 nM (Table 2) (depending on the plant species and cell type (Logan and Knight, 2003; Wagner *et al.*, 2015b; Zottini and Zannoni, 1993)). In mammals, the free matrix Ca^{2+} has been shown to stimulate the activity of several enzymes of the Krebs cycle and the ATP synthase (Bagur and Hajnoczky, 2017). While in mammals mitochondria are essential players of Ca^{2+}-based signalling processes, in plant cells a clear-cut, unambiguous evidence demonstrating the involvement of this organelle in Ca^{2+} signalling processes is still lacking.

The recent use of Ca^{2+} sensors (Rhod-2; aequorin and Cameleon) targeted to the plant mitochondrial matrix (Table 1) have allowed to study *in vivo* the mitochondrial Ca^{2+} dynamics in both resting conditions, and after challenging the plant cells with different stimuli or drug treatments (Logan and Knight, 2003; Loro *et al.*, 2012). A side by side use of transgenic plants stably expressing genetically encoded Ca^{2+} sensors targeted to cytosol or mitochondria have then enabled to define the relationship, in terms of Ca^{2+} handling, among these two different compartments. An important and fundamental finding resulting from these *in vivo* studies was the ability of mitochondria to accumulate and release Ca^{2+} following cytosolic Ca^{2+} transients, being essentially dependent on them (Logan and Knight, 2003; Loro *et al.*, 2012; Manzoor *et al.*, 2012; Teardo *et al.*, 2015; Wagner *et al.*, 2015a). Moreover, stimuli which induce different cytosolic Ca^{2+} increases, in terms of dynamics and magnitudes, were also able to generate different mitochondrial Ca^{2+} dynamics, again confirming the existence of a strict relationship between cytosol and mitochondria. Intriguingly, these works highlighted that mitochondria show slower dynamics of Ca^{2+}accumulation and release with respect to the cytoplasmic variations, strongly pointing to the possibility that plant mitochondria operate as cytosolic Ca^{2+} capacitors, at least locally, playing a role in the shaping of cytosolic Ca^{2+} signals (McAinsh and Pittman, 2009).

In mammals, several reports demonstrated a role of mitochondria in cytosolic Ca^{2+} clearing and buffering, thus affecting and regulating Ca^{2+}-based signalling responses (Rizzuto *et al.*, 2012). However, indications that this way of regulation operates also in plant cells are still lacking - therefore new experimental strategies to demonstrate the existence, if any, of such a mechanism are needed. In this respect, the recent identification of some of the molecular components responsible for the mitochondrial Ca^{2+} transport across the IMM may be of help to test if the "mitochondria clearing hypothesis" is valid in plant. The molecular identification of the mitochondrial channel uniporter (MCU) (Baughman, 2011; De Stefani *et al.*, 2011) allowed to achieve important steps also in the plant field. In Arabidopsis, AtMCU1 and AtMCU2, homologues of the mammalian MCU were shown to localize to mitochondria and to transport Ca^{2+} when expressed in cell-free or heterologous systems (Teardo *et al.*, 2017; Tsai *et al.*, 2016). However, root mitochondria of the *mcu1* knock-out (KO) Arabidopsis plants showed just a small reduction in the Ca^{2+} uptake rate compared to the wild type *in vivo* (Teardo *et al.*, 2017), pointing to a functional redundancy, in line with the prediction of mitochondrial localization of at least five out the six MCU homologs in Arabidopsis (Stael *et al.*, 2012). Interestingly, these isoforms appear to display tissue-specific distribution (Selles *et al*., 2018), possibly allowing to clarify the role of MCUs in a certain tissue using double/triple KO plants.

Besides the MCUs, other possible routes for mitochondrial Ca^{2+} accumulation also exist *in planta*. Some members of the ionotropic glutamate-like receptor (GLR) family have been shown to transport Ca^{2+} (Ortiz-Ramirez *et al.*, 2017; Tapken *et al.*, 2013; Vincill *et al.*, 2012). In addition to the predominant localization of the Arabidopsis GLR3.5 to plastids, a splicing variant is localized to mitochondria and a *glr3.5* KO mutant showed a reduction of the mitochondrial Ca^{2+} accumulation rate compared to the wild type (Teardo *et al.*, 2015). *A priori*, GLR3.5 might work agonistically with the MCU for the accumulation of Ca^{2+}. Although in Arabidopsis there are no reports showing mitochondrial Ca^{2+} dynamics in transgenic lines that overexpress MCUs, the recently described mutant lacking the mitochondrial Ca^{2+} uptake regulator protein (MICU) that inhibits channel activity, showed an overaccumulation of mitochondrial Ca^{2+} (even in resting conditions) when compared to the wild type (Wagner *et al.*, 2015a), therefore potentially mimicking the effects of MCU overexpression. Moreover, the lack of MICU accelerated the speed of mitochondrial Ca^{2+} accumulation in root tip cells in response to external stimuli. However, cytosolic Ca^{2+} dynamics assayed in the *micu* mutant background did not show significant differences if compared to the wild type, indicating that an increase of mitochondrial Ca^{2+} accumulation does not necessarily boost the cytosolic Ca^{2+} clearing.

In summary, the study of both mitochondrial and cytosolic Ca^{2+} dynamics would be fundamental in plants lacking simultaneously MCU isoforms and GLR3.5 (possibly with an inducible system) to define the role of mitochondria in clearing of cytosolic Ca^{2+} and therefore their role for the regulation of Ca^{2+} signalling. In addition to MCUs and GLR3.5, plant mitochondria may have other routes for Ca^{2+} uptake. Three-mitochondrial adenine nucleotide/phosphate carriers (AtAPC1-3) can transport ATP-Ca in reconstituted liposomes (Lorenz *et al.*, 2015). However, evidence that the ATP-Ca transport in mitochondria occurs *in vivo* is lacking. Thus, it would be extremely interesting to study Ca^{2+} dynamics in mitochondria and cytosol of *apcs* mutants carrying mitochondria- and cytosol-targeted Ca^{2+} probes. Pollen tubes or root hairs, where both Ca^{2+} and ATP are fundamental players for a proper growth (Winship *et al.*, 2016), might represent and especially useful systems for these studies. Indeed, a recent work highlighted the importance for MCU2 in pollen tube development even if it was not clear whether the observed phenotype was dependent or not on an altered mitochondrial or cytosolic Ca^{2+} homeostasis (Selles *et al*., 2018).

It must be mentioned that the experiments presented so far were mainly carried out in Arabidopsis root tip cells and essentially designed to study fast Ca^{2+} dynamics in mitochondria and cytosol. The *mcu1*, *mcu2*, *micu* and *glr3.5* plants showed mild phenotypes like altered mitochondrial morphology, reduced pollen tube germination and growth *in vitro*, accelerated senescence or reduced seedling root lengths that can be somewhat difficult to directly correlate with short-term signalling events. The lack of a strong phenotype in terms of mitochondrial and cytosolic Ca^{2+} dynamics and Ca^{2+}-related signalling events can be explained by the lack of a true null mutant (unable to accumulate Ca^{2+} into mitochondria). Nevertheless, studies at specific developmental stages and in specific organs/tissues or cell types may be of help. In support of this idea, it has previously been demonstrated that the concentration of free Ca^{2+} in mitochondria is higher in the tip of the root hairs (500 nM) than in the shanks (200 nM), hence essentially following the cytosolic Ca^{2+} gradient (Wang *et al.*, 2010). As mentioned above, growing pollen tubes and root hairs, that have both a high demand of metabolic energy and require the establishment of a defined cytosolic tip Ca^{2+} gradient, may represent the most suitable system (Michard *et al.*, 2017). Indeed, the recent demonstration that the *mcu2* shows a phenotype in pollen tubes supports this idea (Selles *et al*., 2018). It might be also interesting to understand if and how mitochondrial Ca^{2+} release can regulate cytosolic Ca^{2+} recovery. In a simplified way, we may hypothesize that the slow decrease of mitochondrial Ca^{2+} might delay the cytosolic Ca^{2+} recovery phase. The Arabidopsis genome contains two genes with homology to the mammalian LETM1, an EF-hand protein proposed to be involved in the export of Ca^{2+} from the mitochondria (Austin *et al.*, 2017; Shao *et al.*, 2016). Both Arabidopsis homologs, LETM1 and LETM2, reside in the IMM and the double knockout mutant is not viable (Zhang *et al.*, 2012). To date there are no data showing Ca^{2+} dynamics in the mitochondria or cytosol (of at least single LETM mutants) and the ion species transported by this protein are a matter of debate even in the mammalian system. Hence, it would be important to analyze the cytosol/mitochondria Ca^{2+} handling relationships in an Arabidopsis mutant lacking both LETMs, possibly by using an inducible silencing system to avoid embryonic lethality.

In order to systematically study the role of mitochondria in the regulation of Ca^{2+} signalling, a forward genetic strategy for the isolation of mutants impaired in the mitochondrial Ca^{2+} homeostasis could be of relevance. In this case the use of molecular imaging coupled with high-throughput screenings and possibly with a relatively simple genetic system (e.g. *Physcomitrella patens* or *Marchantia polymorpha, Chlamydomonas reinhardtii*) could provide a series of potential new candidate genes that could help to elucidate the role of mitochondria in Ca^{2+} signalling processes. A similar approach was pursued by Zhao and co-workers (Zhao *et al.*, 2013) that screened an *Arabidopsis thaliana* T-DNA insertion pool to identify mutants defective in salt stress-induced increases in cytosolic Ca^{2+}. This screening pointed to Actin-Related Protein2 (Arp2) which affected not only the salt-induced cytosolic Ca^{2+} increases, but also mitochondria movement, mitochondrial membrane potential and opening of the cell-death triggering permeability transition pore (PTP). An interesting observation was that the pharmacological block of the mitochondrial PTP opening prevented the cytosolic Ca^{2+} increase, but unfortunately the authors did not provide any direct evidence on altered mitochondrial Ca^{2+} dynamics. Another recent work identified the WRKY15 transcription factor as negative regulator of salt and osmotic stress-tolerance in Arabidopsis (Vanderauwera *et al.*, 2012). Importantly, the authors revealed that the WRKY15 overexpression induced an unfolded protein response which impaired the cytosolic Ca^{2+} homeostasis and affected the mitochondrial retrograde regulation mechanism, *de facto* triggering a stress hypersensitivity. Treatment with CPA that affects the activity of ECAs (see above), promoted mitochondrial responses, placing this organelle at the crossroad of ER stress and general cellular responses. A detailed description of Ca^{2+} dynamics in the mitochondria, ER and cytosol

has not been provided, making it difficult to assign a specific role of mitochondria in the regulation of cytosolic Ca^{2+} under these experimental conditions. In summary, suggested role of mitochondrial Ca^{2+} regulation in the salt and osmotic stress response in these latter works is of high interest and deserves further investigation.

Peroxisomal Ca^{2+} signalling

When discussing the role of organelles in Ca^{2+} signalling, peroxisomes have to be taken into account as well, even if as to date, only few studies addressed this question during the last few years. Peroxisomes are ubiquitous single-membrane-bounded organelles that fulfill essential roles in the cellular metabolism. Differentially from mitochondria, peroxisomes do not have any electron transport chain and, to the best of our knowledge, existence of a membrane potential has not been reported. However, the peroxisomal membrane is impermeable to high molecular weight molecules (>1,000 Da) and specific carriers are expressed in the organelles for the transport of different metabolites (Linka and Esser, 2012; Linka and Weber, 2010). Both mammalian and plant peroxisomes accumulate Ca^{2+} into the lumen in response to stimuli that trigger cytosolic Ca^{2+} increases (Costa *et al.*, 2010; Costa *et al.*, 2013; Lasorsa *et al.*, 2008). The resting intra-peroxisomal luminal Ca^{2+} concentration has been estimated to range between 150 nM and 2 µM (Table 2) (Drago *et al.*, 2008). In mammals, stimuli which induce cytosolic Ca^{2+} increases are followed by slow rises in intraperoxisomal Ca^{2+} that do not require either ATP, membrane potential and H^+ gradient (Drago *et al.*, 2008). In plant cells only two reports showed stimulus-induced peroxisomal Ca^{2+} increase in guard cells and root tip cells (Costa *et al.*, 2010). In both cases, the peroxisomal Ca^{2+} dynamics were like the cytosolic ones, reminescent to what is reported in mammalian cells. From the available results, we can summarize that peroxisomes essentially show an equilibration of the peroxisomal luminal Ca^{2+} with that of the cytosol and only potentially work as an additional cytosolic Ca^{2+} buffer. On the other hand, the catalase 3 (CAT3) controls the H_2O_2 levels in guard cells (Zou *et al.*, 2015), and this regulation is dependent on Ca^{2+} in two different ways - one mediated by calmodulin (Yang and Poovaiah, 2002) that operates in peroxisomes, and one mediated by CPK8 operating in the cytosol (Zhou *et al*., 2015). Hence, a stimulus that induces both a cytosolic and peroxisomal Ca^{2+} increase can activate the same enzyme in different locations, via different mechanisms. Another recent observation reports that the peroxisomal Ca^{2+} is required, via a CaM-dependent mechanism, for protein import and for the normal functionality of peroxisomal enzymes, including antioxidant and photorespiratory enzymes, as well as for nitric oxide production (Corpas and Barroso, 2017). In conclusion, the property of peroxisome to accumulate and release Ca^{2+} into and from the lumen has a functional role in plant cell, however currently we lack information about the identity of possible transporters/channels involved in these fluxes.

The apoplast as a main source of Ca^{2+} in signalling

The apoplast is obviously not an intracellular organelle, however together with the vacuole, the cell wall represents the main Ca^{2+} store in plants cells with an estimated concentration of free Ca^{2+} ranging from

0.33 to 1 mM (Table 2) (Conn and Gilliham, 2010; Stael *et al.*, 2012). Remarkably, the apoplast is considered the first plant compartment encountering environmental signals (Gao *et al.*, 2004) and in support of this there are several evidences which demonstrate that the apoplast represents the primary source for the entry of Ca^{2+} in the cell upon the perception of a given stimulus. In fact, by chelating extracellular Ca^{2+}, using EGTA, BAPTA, or by blocking the plasma membrane non-selective cation channels with La^{3+} or Gd^{3+}, stimuli-induced cytosolic Ca^{2+} increases are strongly reduced if not completely abolished (Ali *et al.*, 2007; Knight *et al.*, 1996; Lamotte *et al.*, 2004; Navazio *et al.*, 2007). Despite of the importance of apoplast in the generation of cytosolic Ca^{2+} increases, a limited number of works have reported *in vivo* direct measurements of apoplastic Ca^{2+}. This is mainly due to the high Ca^{2+} concentration and the low pH of the apoplast which make Ca^{2+} measurements challenging, similarly to what we underlined for the vacuole. However, Gao and colleagues were able to target aequorin to the extracellular space and measure apoplastic Ca^{2+} dynamics in response to cold stress revealing that they were different from the cytosolic ones. Remarkably, the authors also showed that the permanent washout of apoplastic Ca^{2+} determined a continuing aequorin signal decay hence confirming the probe's functionality (Gao *et al.*, 2004). More recently Wang and colleagues have instead used the Oregon Green BAPTA 488 5N dye to demonstrate that leaf cells of the *cngc2* and *cax1cax3* mutants overaccumulate apoplastic Ca^{2+} compared to the wild type, when grown in presence of high external Ca^{2+} in the medium (Wang *et al.*, 2017). Interestingly, the overaccumulation of apoplastic Ca^{2+} in the *cax1/cax3* mutant was previously reported by Conn and co-workers performing X-ray microanalysis (Conn *et al.*, 2011). The fact that CAX1 and CAX3 are tonoplast localised Ca^{2+}/H^{+} exchangers, makes this latter observation of a primary importance since it supports the existence of a potential communication between apoplast and vacuole, which will probably deserve more attention.

Conclusion and perspectives

Although there are common elements in Ca^{2+}-based signal transduction networks in all eukaryotes, unique traits of plant Ca^{2+} signalling derive from both structural features of the plant cell and from differences in the lifestyle and developmental programs of plants. Genetic approaches using mutant plants defective in specific Ca^{2+} transporters/channels, together with pharmacological approaches using Ca^{2+} chelators and/or inhibitors of Ca^{2+} channels differentially distributed across cellular membranes, have elucidated how the different stimulus-specific cytosolic Ca^{2+} signatures often derive from the joint contribution of more than one source of Ca^{2+}. Figure 2. summarizes the different channels/transporters possibly involved in Ca^{2+} fluxes in different intracellular membranes. Cross-talks among cellular compartments, possibly due to structurally close contacts may also affect the ensuing global cytoplasmic Ca^{2+} signal. In this respect, the possible use of optical molecular tweezers (Sparkes, 2016) might be of relevance.

In summary, it is clear that the combination of an increasing understanding of the molecular players and elements underlying plant Ca^{2+} signalling in organelles, together with newly generated detection systems for measuring organellar Ca^{2+} concentrations in intact plants, should provide fruitful grounds for ground-breaking discoveries. The view is emerging, that beside transporters, also intracellular ion channels contribute to fine-tuning of cytoplasmic Ca^{2+} dynamics. In this respect, existing proteomic data for different

organelles (e.g. Prime *et al.*, 2000) might allow the identification of further, new (putative) Ca^{2+}-transport modules and decoders that might play a role in shaping Ca^{2+} homeostasis within the plant cell. One of the greatest challenges in the field is the elucidation of how influx and efflux Ca^{2+} transporters/channels are regulated in a concerted manner to translate specific information into a Ca^{2+} signature.

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Figure legends:

Figure 1. Overview of organelle-targeted genetically encoded Ca2+ indicators. Available probes based on aequorin (Knight *et al*., 1991; Johnson *et al*., 1995; Knight *et al*., 1996; van Der Luit *et al.*, 1999; Logan & Knight, 2003; Mehlmer *et al*., 2012; Ordenes *et al*., 2012; Sello *et al.*, 2018), on Cameleon (Mori *et al*., 2006; Costa *et al*., 2010; Krebs *et al*., 2012; Loro *et al*., 2012; 2013; 2016; Bonza *et al*., 2013), on R-Geco1 (Ngo *et al*., 2014; Keinath *et al*., 2015; Waadt *et al*., 2017) and on GCaMP3 or 6 (Ast *et al*; 2017; Liu et al., 2017; Vincent *et al*., 2017a; 2017b; Waadt *et al*., 2017) used in different cell compartments are summarized. See text and Table 2 for details regarding the Ca^{2+} concentration values within the organelles and Table 1 for the affinities of the above probes for Ca^{2+}.

Figure 2. Organelle-located Ca2+ permeable channels and transporters possibly involved in Ca2+ transport across intracellular membranes. Different channels/transporters putatively involved in Ca^{2+} uptake/release into/from organelles and endomembranes are listed. The proteins involved are cyclic nucleotide-gated channels (CNGCs), glutamate receptor-like channels (GLRs), two-pore channels (TPCs), mechanosensitive channels (MSLs), autoinhibited Ca^{2+}-ATPases (ACAs), ER-type Ca^{2+}-ATPases (ECAs), P1-ATPases (HMA1), mitochondrial Ca2+ uniporter complex (MCUC), Ca^{2+}/H^{+} exchangers (CAX) and cation/Ca2+ exchangers (CCX2). See text for further details.

Table 1. Summary of available genetically encoded Ca^{2+} indicators used in plants. The *in vitro* K_D for Ca^{2+} of the different sensors are those reported in the original works. For the bioluminescent aequorin sensors the reported in vitro K_D values are 13 µM (Kendall *et al*., 1992) and 7.2 µM (Brini *et al*., 1995). Other available recently generated Arabidopsis lines expressing GECO variants of Ca^{2+} sensors are reported in Waadt *et al*., 2017.

Name	Version	Type	Peaks of Excitation/ Emission (mn)	*In vitro* K_D for Ca^{2+}*	Subcellular localisation	References
Cameleon	YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	cytosol and nucleus	Nagai *et al.*, 2004; Mori *et al.*, 2006
	NES-YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	cytosol	Krebs *et al*., 2012
	NLS-YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	nucleus	Krebs *et al*., 2012
	NUP-YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	nucleus	Costa *et al*., 2017
	4mt-YC3.6	Ratiometric	Ex 440/Em	250 nM	mitochondria	Loro *et al*., 2012

		CFP/cpVenus	480/530			
	PM-YC3.6-LTI6b	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	plasma membrane	Krebs *et al.*, 2012; Iwano et al., 2015
	2Bam4-YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	chloroplasts and plastids	Loro *et al.*, 2016
	Nano65	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	65 nM	cytosol and nucleus	Horikawa *et al.*, 2010; Choi *et al.*, 2014
	SP-YC4.6-ER	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	58 nM / 14.4 µM	endoplasmic reticulum	Nagai *et al.*, 2004; Iwano *et al.*, 2009; Tian *et al.*, 2014
	2Bam4-YC4.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	58 nM / 14.4 µM	chloroplasts and plastids	Loro *et al.*, 2016
	4mt-D3cpv	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	600 nM	mitochondria	Loro *et al.*, 2013
	D3cpv-KVK-SKL	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	600 nM	peroxisomes	Palmer *et al.*, 2006; Costa *et al.*, 2010
	TP-D3cpv	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	600 nM	tonoplast	Krebs *et al.*, 2012
	CRT-D4ER	Ratiometric CFP/Citrine	Ex 440/Em 480/530	195 µM	endoplasmic reticulum	Palmer *et al.*, 2006; Bonza *et al.*, 2013
Twitch	Twitch 3	Ratiometric CFP/cpCit174	Ex 440/Em 480/530	250 nM	cytosol and nucleus	Thestrup *et al.*, 2014; Waadt *et al.*, 2017
Geco	R-Geco1	Intensiometric mApple	Ex 561/Em 600	482 nM	cytosol and nucleus	Zhao *et al.*, 2011; Ngo *et al.*, 2014; Keinath *et al.*, 2015
	NR-Geco1	Intensiometric mApple	Ex 561/Em 600	482 nM	nuclear	Zhao *et al.*, 2011; Kelner *et al.*, 2018

	NR-Geco1.2	Intensiometric mApple	Ex 561/Em 600	1.2 µM	nuclear	Wu *et al.*, 2013; Kelner *et al.*, 2018
	CG-Geco1	Intensiometric cpGFP	Ex 488/Em 515	749 nM	cytosol	Zhao *et al.*, 2011; Kelner *et al.*, 2018
	CG-Geco1.2	Intensiometric cpGFP	Ex 488/Em 515	1.15 µM	cytosol	Zhao *et al.*, 2011; Kelner *et al.*, 2018
	R-Geco1-mTurquoise	Ratiometric mApple/mTurqouise	Ex 405/561/Em 480/600	NA	cytosol and nucleus	Waadt *et al.*, 2017
GCaMP	GCaMP3	Intensiometric cpGFP	Ex 488/Em 515	542 nM	cytosol and nucleus	Vincent *et al.*, 2017a; 2017b
	GCaMP6f	Intensiometric cpGFP	Ex 488/Em 515	375 nM	cytosol and nucleus	Waadt *et al.*, 2017
	GCaMP6s	Intensiometric cpGFP	Ex 488/Em 515	144 nM	cytosol and nucleus	Liu *et al.*, 2017
	MatryoshCaMP6s	Ratiometric cpGFP/LSSmOrange	Ex 440/Em 515/600	197 nM	cytosol and nucleus	Ast *et al.*, 2017
Case	Case12	Intensiometric cpGFP	Ex 488/Em 515	1 µM	cytosol and nucleus	Souslova *et al.*, 2007; Zhu *et al.*, 2013
Aequorin	Aequorin	Bioluminescence	No Ex -/Em 465	7.2-13 µM	cytosol and nucleus	Knight *et al.*, 1991
	Aequorin	Bioluminescence	No Ex -/Em 465	7.2-13 µM	nucleus	van Der Luit *et al.*, 1999
	Aequorin	Bioluminescence	No Ex -/Em 465	7.2-13 µM	chloroplast stroma	Johnson *et al.*, 1995
	Aequorin	Bioluminescence	No Ex -/Em 465	7.2-13 µM	mitochondria	Logan and Knight, 2003
	Aequorin	Bioluminescence	No Ex -/Em 465	7.2-13 µM	Golgi	Ordenes *et al.*, 2012
	Aequorin	Bioluminescence	No Ex -/Em 465	7.2-13 µM	vacuole/tonoplast	Knight *et al.*, 1996

YFP-aequorin	CYA	Bioluminescence	No Ex -/Em 465	7.2-13 µM	cytosol	Mehlmer *et al.*, 2012
	NYA	Bioluminescence	No Ex -/Em 465	7.2-13 µM	nucleus	Mehlmer *et al.*, 2012
	YA	Bioluminescence	No Ex -/Em 465	7.2-13 µM	plasma membrane	Mehlmer *et al.*, 2012
	CHYA	Bioluminescence	No Ex -/Em 465	7.2-13 µM	chloroplast/plastid stroma	Mehlmer *et al.*, 2012; Sello *et al.*, 2016
	MYA	Bioluminescence	No Ex -/Em 465	7.2-13 µM	mitochondria	Mehlmer *et al.*, 2012
	OEYA	Bioluminescence	No Ex -/Em 465	7.2-13 µM	chloroplast outer envelope	Mehlmer *et al.*, 2012; Sello *et al.*, 2016
	IEYA	Bioluminescence	No Ex -/Em 465	7.2-13 µM	chloroplast inner envelope	Mehlmer *et al.*, 2012
	TL-YA	Bioluminescence	No Ex -/Em 465	7.2-13 µM	chloroplast thylakoid lumen	Sello *et al.*, 2018
	TM-YA	Bioluminescence	No Ex -/Em 465	7.2-13 µM	chloroplast thylakoid membrane	Sello *et al.*, 2018
GFP5-aequorin	pchitGFP5:AQ	Bioluminescence	No Ex -/Em 465	NA	apoplast	Gao *et al.*, 2004
GFP-aequorin	G5A	Bioluminescence resonance energy transfer	No Ex -/Em 515	NA	cytosol and nucleus	Baubet *et al.*, 2000; Xiong *et al.*, 2014

Table 2. Summary of measured and estimated Ca^{2+} concentrations at resting conditions in the different subcellular compartments of plant cells. The reported values are an estimation of Ca^{2+} concentrations in the different compartments based on direct measurements or deducted from the in vitro K_D of the Ca^{2+} sensors reported in Table 1.

Subcellular compartment	Range/estimation of resting free Ca^{2+}	Method of measurement	References
Apoplast	330 µM - 1 mM	X-ray microanalysis	Conn *et al*., 2011
Cytosol	50 - 100 nM	Aequorin, Cameleon (YC3.6), R-Geco1	Knight and Knight, 1995, Logan and Knight, 2003; Wagner *et al*., 2015a; Waadt *et al*., 2017
Nucleus	100 nM	Aequorin	Van der Luit et al., 1999; Mithöfer and Mazars, 2002
Mitochondria matrix	100 - 600 nM	Aequorin, Cameleon (YC3.6), Fura-2	Zottni and Zannoni, 1993; Logan and Knight, 2003; Wagner *et al*., 2015a;
Chloroplast stroma	100 - 200 nM	Aequorin, Cameleon (YC3.6)	Mehlmer *et al*., 2012; Nomura *et al*., 2012; Sello *et al*., 2016; Loro *et al*., 2016
Thylakoid lumen	500 nM	Aequorin	Sello *et al*., 2018
Amyloplast/Plastid stroma	80 - 100 nM	Aequorin, Cameleon (YC3.6)	Sello *et al*., 2016; Loro *et al*., 2016
Vacuolar lumen	200 µM - 50 mM	X-ray microanalysis	Conn and Gilliham, 2010; Conn *et al*., 2011
Endoplasmic reticulum lumen	50 - 500 µM	Cameleon (CRT-D4ER)	Iwano et al., 2009; Bonza *et al*., 2013
Golgi lumen	700 nM	Aequorin	Ordenes *et al*., 2012
Peroxisome lumen	150 nM - 2 µM	Cameleon (D3cpv-KVK-SKL)	Costa *et al*., 2010