The contribution of organelles to plant intracellular calcium signalling

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Abstract

Calcium (Ca$^{2+}$) is among the most important intracellular messengers in living organisms. Understanding 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 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.

Keywords: Aequorin, calcium-permeable channels, calcium-permeable transporters, Cameleon, endomembranes, genetically encoded calcium indicators, organelle

Introduction

Changes in Ca$^{2+}$ levels within plant cells can be considered as hallmarks of a plethora of processes such as growth, differentiation, regulation of stomatal opening, induction of pathogen defence responses, establishment of plant–microbe symbioses, and stress adaptation. Indeed, each of these processes is 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, for example, Trewavas et al., 1996; Evans et al., 2001; Xiong et al., 2006; Monshausen, 2012; Whalley and Knight, 2013). 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 sustained increases above approximately 10$^{-4}$ M can lead to protein and nucleic acid aggregation and to precipitation of phosphates, thus causing damage to membranes and organelles, ultimately leading to a generalized cytotoxicity. On the other hand, rapid and transient increases of the 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, $[Ca^{2+}]_{\text{cyt}}$ increases from $\sim 10^{-7}$ M to $10^{-6}$ M, relaying an external stimulus to the intracellular milieu and allowing triggering of 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 $[Ca^{2+}]_{\text{cyt}}$ (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 (Stael et al., 2012; Nomura and Shiina, 2014). 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 (Trewavas et al., 1996; Kudla et al., 2010). $Ca^{2+}$ can be mobilized from storage compartments such as the cell wall/apoplast, the vacuole, and the endoplasmic reticulum (ER), whereas the nucleus, as well as chloroplasts and mitochondria, can also generate intracellular $Ca^{2+}$ 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 oversimplified linear pathways (with a stimulus generating a transient $[Ca^{2+}]_{\text{cyt}}$ 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 $[Ca^{2+}]_{\text{cyt}}$—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 the bioenergetic organelles mitochondria and chloroplasts, as well as to peroxisomes and the ER, whereas we advise 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 in the $Ca^{2+}$ signalling network (Charpentier, 2018).

**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 increases in the $Ca^{2+}$ level (e.g. Tang and Luan, 2017).

$Ca^{2+}$ signatures are decoded by $Ca^{2+}$-binding sensor proteins that act either as primary responders or as signal relays (DeFalco et al., 2009; Zhu, 2016; Tang and Luan, 2017). $Ca^{2+}$-dependent protein kinases (CDPKs) 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 development (see, for example, Lenzoni et al., 2018). Excellent, recent reviews underline the crucial role of the above protein families in global $Ca^{2+}$ signalling (Ranty et al., 2016; Simeunovic et al., 2016; Tang and Luan, 2017; Kudla et al., 2018), therefore the present review only briefly mentions 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/$Ca^{2+}$ exchangers (CCXs) that are emerging players in an increasing range of cellular and physiological functions (Bose et al., 2011; Frei et al. 2012; Pittman and Hirschi, 2016; Costa et al., 2017; Corso et al., 2018). $Ca^{2+}$-permeable channels include members of the glutamate-like receptor family (Swarbreck et al., 2013; Forde and Roberts, 2014; Steinhorst and Kudla, 2014), of cyclic nucleotide-gated channels (CNGCs) (Dietrich et al., 2010; DeFalco et al., 2016), and of mechanosensitive channels (MSCs) (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 channel TPC1 (Petter et al., 2005; Choi et al., 2014, 2017; Kiep et al., 2015; Vincent et al., 2017a; Hedrich et al., 2018) and the mitochondrial calcium uniporter (Wagner et al., 2015a, 2016; Teardo et al., 2017) 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 (McAinsh et al., 1995; Ehrhardt et al., 1996; Holdaway-Clarke et al., 1997). The use of these dyes allowed fundamental discoveries to be made, but they present some limitations due to their requirement to be loaded or manually injected and also because their use suffers from low throughput and variability, and is prone to artefacts. 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 (GECIs) (Pérez 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 subcellular compartments by specific targeting of GECIs to organelles (Stael et al., 2012; Costa and Kudla, 2015). Furthermore, the possibility to calibrate GECIs may allow information on absolute concentrations for different ions to be obtained (see, for
example, Lanquar et al., 2014 for Zn$^{2+}$ and Waadt et al., 2017 for Ca$^{2+}$).

The first subcompartamental (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 organelar Ca$^{2+}$ changes in the animal field (Brini et al., 1999; Rudolf et al., 2003; Ottolini et al., 2014; Bagur and Hajnoczky, 2017) 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, 1998).

In plants, thus far the two main Ca$^{2+}$ indicators used are aequorin and Cameleon (Knight and Knight, 1995; Mithöfer and Mazzars, 2002; Costa and Kudla, 2015) and, importantly, both display a binding affinity for Ca$^{2+}$ that renders them useful to detect changes in Ca$^{2+}$ concentrations in the ranges that occur physiologically (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, 1992; Sai and Johnson, 2002; Logan and Knight, 2003). 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 make it an excellent tool to measure Ca$^{2+}$ levels in chlorophyll-containing tissues even for long time intervals (Martí 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, namely 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 green fluorescent protein (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 visualization of 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 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 harbour cyan and yellow fluorescent proteins (CFP and YFP, or spectral variants thereof) linked together by the Ca$^{2+}$-binding protein 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 a reduced distance between CFP and YFP and an 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. Cameleon sensors are available for different intracellular compartments and even for simultaneous measurement of Ca$^{2+}$ dynamics in different subcellular compartments (Krebs et al., 2012; Costa and Kudla, 2015).

Other GFP-based Ca$^{2+}$ biosensors, such as, for example, Case12, GCAmp3, GCAmp6 (Zhu et al., 2014; Liu et al., 2017; Vincent et al., 2017a, b), 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 of Ca$^{2+}$ in the cytosol and nucleus (Ngo et al., 2014; Keinath et al., 2015; Waadt et al., 2017; Kelner et al., 2018). Furthermore, a strategy of a novel, dual-FP (fluorescent protein) biosensor with large dynamic range based on employment of a single FP-cassette that nests a stable reference FP (large Stokes shift LSSmOrange) within a reporter FP (circularly permutated GFP) 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 with 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-FP 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 apply distinct Ca$^{2+}$ probes localized to different compartments simultaneously, 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-Gec1 and NR–Gec1 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
Table 1. Summary of available genetically encoded Ca\(^{2+}\) indicators used in plants

<table>
<thead>
<tr>
<th>Name</th>
<th>Version</th>
<th>Type</th>
<th>Peaks of excitation/ emission (nm)</th>
<th>In vitro K(_{o}) for Ca(^{2+})</th>
<th>Subcellular localization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cameleon</td>
<td>YC3.6</td>
<td>Ratiometric CFP/cpVenus</td>
<td>Ex 440/Em 480/530 250 nM</td>
<td>Cytosol and nucleus</td>
<td>Nagai et al. (2004); Mori et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>NES-YC3.6</td>
<td></td>
<td>Ratiometric CFP/cpVenus</td>
<td>Ex 440/Em 480/530 250 nM</td>
<td>Cytosol</td>
<td>Krebs et al. (2012)</td>
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<tr>
<td>NLS-YC3.6</td>
<td></td>
<td>Ratiometric CFP/cpVenus</td>
<td>Ex 440/Em 480/530 250 nM</td>
<td>Nucleus</td>
<td>Krebs et al. (2012)</td>
<td></td>
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<td>NUP-YC3.6</td>
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<td>Ex 440/Em 480/530 250 nM</td>
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<td>Costa et al. (2017)</td>
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</tr>
<tr>
<td>4mt-YC3.6</td>
<td></td>
<td>Ratiometric CFP/cpVenus</td>
<td>Ex 440/Em 480/530 250 nM</td>
<td>Mitochondria</td>
<td>Loro et al. (2012)</td>
<td></td>
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<tr>
<td>PM-YC3.6,LT16b</td>
<td></td>
<td>Ratiometric CFP/cpVenus</td>
<td>Ex 440/Em 480/530 250 nM</td>
<td>Plasma membrane</td>
<td>Krebs et al. (2012); Ivano et al. (2015)</td>
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<tr>
<td>2Bam4-YC3.6</td>
<td></td>
<td>Ratiometric CFP/cpVenus</td>
<td>Ex 440/Em 480/530 250 nM</td>
<td>Chloroplasts and plastids</td>
<td>Loro et al. (2016)</td>
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<td>SP-YC4.6-ER</td>
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<td>Ratiometric CFP/cpVenus</td>
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<td>Endoplasmic reticulum</td>
<td>Nagai et al. (2004); Ivano et al. (2009);</td>
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<td>Tian et al. (2014)</td>
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<td>Ex 440/Em 480/530 58 nM/14.4 μM</td>
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<td>Loro et al. (2016)</td>
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<td>4mt-D3cpv</td>
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<td>Ex 440/Em 480/530 600 nM</td>
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<td>D3cpv-KVK-SKL</td>
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<td>Tonoplast</td>
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<td>CRT-D4ER</td>
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<td>Twitch 3</td>
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<td>Geco</td>
<td>R-Geco1</td>
<td>Intensiometric mApple</td>
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<td>Zhao et al. (2011); Ngo et al. (2014); Keinath et al. (2015)</td>
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<td>Wu et al. (2013); Kelner et al. (2018)</td>
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<td>Ex 488/Em 515 749 nM</td>
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<td>GCaMP3</td>
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<td>Aequorin</td>
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<td>Chloroplast/plastid stroma</td>
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<td>Mitochondria</td>
<td>Mehmer et al. (2012)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OEA</td>
<td>Bioluminescence</td>
<td>No Ex/Em 465 7.2–13 μM</td>
<td>Chloroplast outer envelope</td>
<td>Mehmer et al. (2012)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IEYA</td>
<td>Bioluminescence</td>
<td>No Ex/Em 465 7.2–13 μM</td>
<td>Chloroplast inner envelope</td>
<td>Mehmer et al. (2012)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TL-YA</td>
<td>Bioluminescence</td>
<td>No Ex/Em 465 7.2–13 μM</td>
<td>Chloroplast thylakoid lumen</td>
<td>Sello et al. (2018)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TM-YA</td>
<td>Bioluminescence</td>
<td>No Ex/Em 465 7.2–13 μM</td>
<td>Chloroplast thylakoid membrane</td>
<td>Sello et al. (2018)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEP5–aequorin</td>
<td>pchitGFP5/AQ</td>
<td>Bioluminescence resonance energy transfer</td>
<td>No Ex/Em 465 7.2–13 μM</td>
<td>Apoplast</td>
<td>Gao et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>GFP–aequorin</td>
<td>GSA</td>
<td>Bioluminescence resonance energy transfer</td>
<td>No Ex/Em 465 7.2–13 μM</td>
<td>Cytosol and nucleus</td>
<td>Baubet et al. (2003); Xiong et al. (2014)</td>
</tr>
</tbody>
</table>

The in vitro K\(_{o}\) values for Ca\(^{2+}\) of the different sensors are those reported in the original works. For the bioluminescent aequorin sensors, the reported in vitro K\(_{o}\) 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).
Organelles and endomembranes as calcium signal regulators

As mentioned above, organelle-targeted, bioluminescent or fluorescent GECIs, summarized in Fig. 1, have greatly advanced the field of organelar 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 for Ca^{2+} (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 20–40 µm, occupying 80–90% of the cell volume in mature plant cells. Rather than being just the plant counterparts of animal lysosomes, they actually fulfil 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,
Table 2. Summary of measured and estimated Ca$^{2+}$ concentrations at resting conditions in the different subcellular compartments of plant cells

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

The reported values are an estimation of Ca$^{2+}$ concentrations in the different compartments based on direct measurements or deduced from the in vitro $K_0$ of the Ca$^{2+}$ sensors reported in Table 1.

A Cameleon-based tonoplast-targeted sensor has also been generated, but, besides labelling the tonoplast, it was also present in the cytosol, restricting the actual usefulness of such a 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 make difficult to use the currently available GECIs to monitor Ca$^{2+}$ dynamics efficiently 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., 2018) 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 (Martinoto et al., 2012; Xu et al., 2015). Many of these transport systems have been molecularly identified during the last few decades (Martinoto et al., 2012; Neuhaus and Trentmann, 2014). Ca$^{2+}$ is taken up into the vacuole probably by two P-type Ca$^{2+}$ pumps, such as CaM-regulated autoinhibited Ca$^{2+}$-ATPases (ACAAs), as well as by Ca$^{2+}$/proton exchangers (CAXs) (Edel et al., 2017), which exhibit a high sequence homology to their yeast counterparts also residing on the vacuolar membrane (Hirschi et al., 1994). ACA pumps exist in at least 10 isoforms in Arabidopsis (Geisler et al., 2000). The 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 (PCD) pathway in plants (Boursiac et al., 2010). Among the six CAX members in Arabidopsis, AtCAX1–AtCAX4 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 a low expression level, plays an important role 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; Martinoto et al., 2012; Socha and Guerinot, 2014; Pittman and Hirschi, 2016).

The vacuole, together with the cell wall/apoplast, is 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 (InsP3) releases Ca$^{2+}$ predominantly from the vacuole (Alexandre and Lassalles, 1990; Allen et al., 1995). However, later experiments indicated that in plants, inositol-hexakisphosphate (InsP6) plays a prominent role with respect to InsP3 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. Peiter et al., 2005; Carpaneto and Gradogna, 2018), whose structure has been solved (Guo et al., 2016), acts as a tonoplast channel. Since the physiological concentration of K$^+$ both in the cytosol and inside the vacuole (~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\textsuperscript{2+} detection by fluorescence finally led to the demonstration that Ca\textsuperscript{2+} is able to permeate through TPC1, even if its concentration (0.5 mM) is much lower than that of K\textsuperscript{+} (105 mM) in electrophysiological experiments (Gradogna et al., 2009; Carpenato and Gradogna, 2018). Plants lacking TPC1 are defective in both ABA–induced repression of germination and in the response of stomata to extracellular Ca\textsuperscript{2+}, demonstrating a critical role for the vacuole Ca\textsuperscript{2+} release channel in various physiological processes of plants (Peiter et al., 2005). Furthermore, the propagation of salt stress-induced long-distance Ca\textsuperscript{2+} waves as well as wounding/herbivory–triggered Ca\textsuperscript{2+} waves was found to be dependent on TPC1 in Arabidopsis (Choi et al., 2014; Kiep et al., 2015). However, other reports dismiss a prominent role for TPC1 in vacuolar Ca\textsuperscript{2+} release, assessed either by equorin or by direct patch-clamp analyses. For example, unaltered cytosolic Ca\textsuperscript{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\textsuperscript{2+} gradients, TPC1 was shown to conduct Ca\textsuperscript{2+} into the vacuole, suggesting that it dissipates rather than generates cytosolic Ca\textsuperscript{2+} signals (at least during external Ca\textsuperscript{2+}–induced stomatal closure) (Rienmüller et al., 2010). Likewise, the findings that the fou2 mutant plants harbouring a hyperactive TPC1 channel variant (D454N) show an increased vacuolar Ca\textsuperscript{2+} content (Beyhl et al., 2009) and a decreased resting cytosolic Ca\textsuperscript{2+} level compared with the wild type argue against a Ca\textsuperscript{2+} release function of TPC1. The fou2 mutant also has a slightly lower resting cytosolic Ca\textsuperscript{2+} compared with the wild type, and cytosolic Ca\textsuperscript{2+} increases after wounding were found to be similar in both plants (Lenglet et al., 2017). On the other hand, a recent study, carried out by using the fluorescent Ca\textsuperscript{2+} biosensor GCaMP3, highlighted a functionally relevant interplay between the plant defence co-receptor Brassinosteroid insensitive–associated kinase1 (BAK1), the PM-localized glutamate receptors GLR3.3 and GLR3.6, and TPC1 to mediate cytosolic Ca\textsuperscript{2+} elevations following biotic stress such as aphid attack (Vincent et al., 2017a). Interestingly, another study highlighted the importance of endomembrane cation fluxes in controlling the basal level of the wound-inducible defence 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\textsuperscript{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 (Hedrich, 2012; Martinoia et al., 2012; Edel et al., 2017; Francisco and Martinoia, 2018).

A role for the endoplasmic reticulum in plant intracellular Ca\textsuperscript{2+} signalling?

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

The involvement of the ER in Ca\textsuperscript{2+} homeostasis and signalling in plant cells has long been underappreciated, possibly overshadowed by the prominent role commonly ascribed to the vacuole, and because of the lack, for a long time, of direct measurements of luminal [Ca\textsuperscript{2+}] ([Ca\textsuperscript{2+}]\textsubscript{ER}) and its potential variations during signal transduction. Functional conservation of calreticulin as the major high–capacity (15–30 mol of Ca\textsuperscript{2+} per mol of protein), low-affinity (K\textsubscript{D}=0.5 mM) Ca\textsuperscript{2+}–binding protein in the lumen of the plant ER (for reviews, see, for example, Mariani et al., 2003; Jia et al., 2009) has provided circumstantial evidence for submillimolar [Ca\textsuperscript{2+}]\textsubscript{ER}. In addition to ER Ca\textsuperscript{2+} storage and modulation of Ca\textsuperscript{2+} homeostasis, calreticulin has been shown to function, together with calnexin, as a 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\textsuperscript{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\textsubscript{D}s 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\textsuperscript{2+}]\textsubscript{ER} gradient required for pollen tube growth (Iwano et al., 2009).

Arabidopsis contains four P(IIA)-type ATPase genes, AtECA1–AtECA4, which are expressed in all major organs of Arabidopsis. ECA1 knock-out mutants grew poorly on medium with low Ca\textsuperscript{2+} or high Mn\textsuperscript{2+}, indicating that ECA1-mediated uptake of these divalent cations into the ER is required for plant growth under conditions of Mn\textsuperscript{2+} toxicity or Ca\textsuperscript{2+} deficiency (Wu et al., 2002). The silencing of an ER-localized type IIB Ca\textsuperscript{2+}–ATPase (ACA like) in tobacco has been found to alter intracellular Ca\textsuperscript{2+} signalling and accelerate PCD during the plant innate immune response, indicating that the Ca\textsuperscript{2+} uptake pathway into the ER functions as a regulator of PCD (Zhu et al., 2010). Ca\textsuperscript{2+} release from the ER has also been proposed to play an essential role in sieve tube occlusion via Ca\textsuperscript{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\textsubscript{D} of 195 μM) (Table 1), allowed the dynamic, in vivo monitoring of ER luminal Ca\textsuperscript{2+}, showing that the ER may also work as a capacitor/buffer of cytosolic Ca\textsuperscript{2+} transients (Bontza et al., 2013). In fact, cytosolic Ca\textsuperscript{2+} increases triggered by different stimuli (salt stress, external ATP and glutamate) were followed by Ca\textsuperscript{2+} accumulation into the ER lumen, but not by release. Moreover, dynamically, the ER Ca\textsuperscript{2+} increases followed temporally the cytosolic increases, showing slower rate of accumulation and release (Bontza et al., 2013; Corso et al., 2018). Another clue in favour of the role of the ER, as a cytosolic Ca\textsuperscript{2+} capacitor is confirmed by the effect of cyclopiazonic acid (CPA) (an inhibitor of IIA Ca\textsuperscript{2+}–ATPase ECA) which reduced the luminal ER Ca\textsuperscript{2+} concentration and increased that in the cytosol (Zappini et al., 2004; Bontza et al., 2013), indicating the ECAs as fundamental players for ER Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+}-permeable channels located in higher plant ERs, early biochemical studies have indicated the occurrence of ER Ca\textsuperscript{2+}-mobilization pathways activated by voltage (Klüssener et al., 1995) and by two structurally related molecules, namely the pyridine nucleotide derivatives nicotinic adenine dinucleotide phosphate (NAADP) (Navazio et al., 2000) and cyclic ADP-ribose (cADPR) (Navazo et al., 2001). The molecular identity of the above voltage- and ligand-gated Ca\textsuperscript{2+}-permeable channels, however, has not been unravelled yet.

The ER has a unique architecture that facilitates the spatiotemporal segregation of biochemical reactions and the establishment of interorganelar communication networks. Spatially confined ER–PM microdomains are emerging as highly specialized signalling hubs both in animal systems (e.g. Son et al., 2016; Demaurex and Guido, 2017) and in plants (Bayer et al., 2017). In addition, the continuity between ER membranes and the outer nuclear membrane suggests a potential role for the ER as a Ca\textsuperscript{2+} store participating in the repetitive Ca\textsuperscript{2+} release/uptake from the nucleoplasm and perinuclear cytosol during legume symbioses (Capoën et al., 2011). CNGCs have recently been demonstrated to mediate these nuclear-associated Ca\textsuperscript{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 the ER and plastids may exchange not only lipids, but also ions such as Ca\textsuperscript{2+}, opens up the possibility of a complex and finely tuned Ca\textsuperscript{2+} regulation, involving potential ER–plastid crosstalk (Mehrshahi et al., 2013). On the other hand, the role of ER–mitochondria contact sites in shaping the cytosolic Ca\textsuperscript{2+} signalling is well documented in mammals (e.g. Rizzuto et al., 2012; Brini et al., 2018), but not in plants. We can envisage such an intimate liaison also in the case of plant cells, although direct proof is missing in this case.

### The plant Golgi apparatus: a rather unexplored Ca\textsuperscript{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 micrometres per second) along the surface of the ER (Robinson et al., 2015). In addition to essential roles in protein glycosylation and trafficking (Vitale and Galli, 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\textsuperscript{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\textsuperscript{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 Ca\textsuperscript{2+} handling by this compartment may also exhibit some specificity in plant cells. Compared with the extensive information about Ca\textsuperscript{2+} handling by the Golgi in mammalian cells (for a review, see Pizzo et al., 2011), knowledge about Ca\textsuperscript{2+} homeostasis and signalling in the plant Golgi is still scarce. Free Ca\textsuperscript{2+} levels in the Golgi ([Ca\textsuperscript{2+}]\textsubscript{Golgi}) were estimated to be ~0.70 μM (Table 2) (Ordenes et al., 2012), a value which is much lower than [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}-buffering systems inside the Golgi, and indeed calreticulin has been reported to be localized at the plant Golgi, in addition to the ER (Navazio et al., 2002; Nardi et al., 2006). Interestingly, transient increases in Ca\textsuperscript{2+} dynamics were observed in response to several abiotic stimuli, such as cold shock, mechanical stimulation, and hypertonic stress, whereas the administration of the synthetic auxin analogue 2,4-dichlorophenoxy acetic acid (2,4-D) induced a slow decrease of organelar Ca\textsuperscript{2+} (Ordenes et al., 2012). Concerning Ca\textsuperscript{2+} decoding mechanisms, two calmodulin-like proteins from Arabidopsis 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\textsuperscript{2+} signals (Ruge et al., 2016). The nature of Ca\textsuperscript{2+}-transporting proteins still awaits clarification. Among the four IIA Ca\textsuperscript{2+}-ATPase in Arabidopsis, AtECa3 was proposed to function in the transport of Ca\textsuperscript{2+} and Mn\textsuperscript{2+} ions into the Golgi (Mills et al., 2008).

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

### Chloroplasts as Ca\textsuperscript{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\textsuperscript{2+} signals (reviewed by McAnish and Pittman, 2009; Rocha and Vothknecht, 2012; Nomura and Shina, 2014; Kmiecik et al., 2016). Chloroplasts, which possess a high concentration of Ca\textsuperscript{2+}, serve as important intracellular cytosolic Ca\textsuperscript{2+} capacitors in plant cells, and they may also influence the entire cellular Ca\textsuperscript{2+} network by modulating cytosolic Ca\textsuperscript{2+} transients. Thus, they can contribute to shaping cytoplasmic Ca\textsuperscript{2+} signatures (Nomura et al., 2012; Loro et al., 2016; Sello et al., 2016).

The predominant portion of the chloroplastic Ca\textsuperscript{2+} (~15 nM) is bound to the negatively charged thylakoid membranes or to Ca\textsuperscript{2+}-binding proteins, keeping the resting free [Ca\textsuperscript{2+}]\textsubscript{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 (Mehlner et al., 2012). We have recently used these plastid-targeted aequorin probes to reveal differential stimulus–specific Ca$^{2+}$ responses of anlyoplasts versus chloroplasts (Sello et al., 2016), suggesting that Ca$^{2+}$ signalling might have specific roles during plastid development. Interestingly, using a 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) remains elusive (for recent reviews, see, for example, Finazzi et al., 2015; Carraretto et al., 2016; Pottosin and Shabala, 2016). 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 (Szabo and Zoratti, 2014; Carraretto et al., 2016), the most promising inner envelope–located candidate includes 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 (Tardeo et al., 2010, 2011) and GLR3.5 (Tardeo et al., 2015) and the mechano-sensitive 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) are also candidates for mediating Ca$^{2+}$ flux across the inner envelope membrane. The possible role, localization (for ACA1), and specificity of the latter two proteins are, 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 homologue of PAM71, PAM71–HL, is located to the chloroplast envelope and is likely to exert the same function (Schneider et al., 2016; Eisenhut et al., 2018), as the homologues 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$^{+}$ antipporter with critical functions in the regulation of PSII and in chloroplast Ca$^{2+}$ and pH homeostasis in Arabidopsis (Wang et al., 2016). The possibility that this protein is able to transport manganese in a Ca$^{2+}$–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 homologues 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 have not been described up to now, in contrast to four other mitochondria–located AtMCU homologues (Wagner et al., 2015a; Carraretto et al., 2016; Tardeo et al., 2017). At present, it is difficult to understand whether the FAAC 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 et al. (2016) provided 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 contribute critically to Ca$^{2+}$ homeostasis (Rocha and Vothknecht, 2012; Stael et al., 2012; Hochmal et al., 2015). 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, the thylakoid–located Post–Floral–specific gene 1 PPF1, and the glycosyltransferase QUASIMODO1 (QUA1) (Wang et al., 2003; Nomura et al., 2008; Petrovtsos et al., 2011; 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 yet been fully elucidated (Wang et al., 2012, 2016; Fu et al., 2013). 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 defence-related genes was shown to be dependent on CAS. Furthermore, the activity of mitogen-activated protein kinases (MAPK) 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., 2016; 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 (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 also to occur 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 measurement of Ca\(^{2+}\) concentrations inside and around thylakoids (Table 2) and to monitor dynamic Ca\(^{2+}\) changes in the above subchloroplast 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 highlight further 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 (Kigle 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 Shinaia, 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 ~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 synthesize 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, such as Ca\(^{2+}\), which flux into the matrix passively through channels, reaching free Ca\(^{2+}\) concentrations with values ranging from 100 nM to 600 nM (Table 2) (depending on the plant species and cell type; Zottini et al., 2008, 2012; Manzoor et al., 2015b). 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 Hajnóczky, 2017). While in mammalian mitochondria are essential players of Ca\(^{2+}\)-based signalling processes, in plant cells 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 study in vivo of the mitochondrial Ca\(^{2+}\) dynamics both in 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 the cytosol or mitochondria has then enabled the relationship, in terms of Ca\(^{2+}\) handling among these two different compartments, to be defined. 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 the cytosol and mitochondria. Intriguingly, these works highlighted that mitochondria show
slower dynamics of Ca2+ accumulation and release with respect to the cytoplasmic variations, strongly pointing to the possibility that plant mitochondria operate as cytosolic Ca2+ capacitors, at least locally, playing a role in the shaping of cytosolic Ca2+ signals (McAinsh and Pittman, 2009).

In mammals, several reports demonstrated a role for mitochondria in cytosolic Ca2+ clearing and buffering, thus affecting and regulating Ca2+-based signalling responses (Rizzuto et al., 2012). However, indications that this mode of regulation also operates 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 Ca2+ transport across the IMM may be of help to test if the ‘mitochondria clearing hypothesis’ is valid in plants. The molecular identification of the MCU (Baughman et al., 2011; De Stefani et al., 2011) allowed important steps in the plant field also to be achieved. In Arabidopsis, AtMCU1 and AtMCU2, homologues of the mammalian MCU, were shown to localize to mitochondria and to transport Ca2+ when expressed in cell-free or heterologous systems (Tsai et al., 2016; Teardo et al., 2017). However, root mitochondria of the mcu1 knock-out (KO) Arabidopsis plants showed just a small reduction in the Ca2+ uptake rate compared with 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 homologues in Arabidopsis (Stael et al., 2012). Interestingly, these isoforms appear to display tissue-specific distribution (Selles et al., 2018), possibly allowing clarification of the role of MCUs in a certain tissue using double/triple KO plants.

Besides the MCUs, other possible routes for mitochondrial Ca2+ accumulation also exist in planta. Some members of the ionotropic glutamate-like receptor (GLR) family have been shown to transport Ca2+ (Vincill et al., 2012; Tapken et al., 2013; Ortiz-Ramírez et al., 2017). 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 Ca2+ accumulation rate compared with the wild type (Teardo et al., 2015). A priori, GLR3.5 might work agonistically with the MCU for the accumulation of Ca2+. Although in Arabidopsis there are no reports showing mitochondrial Ca2+ dynamics in transgenic lines that overexpress MCUs, the recently described mutant lacking the mitochondrial Ca2+ uptake regulator protein (MICU) that inhibits channel activity showed an overaccumulation of mitochondrial Ca2+ (even in resting conditions) when compared with 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 Ca2+ accumulation in root tip cells in response to external stimuli. However, cytosolic Ca2+ dynamics assayed in the muc1 mutant background did not show significant differences if compared with the wild type, indicating that an increase of mitochondrial Ca2+ accumulation does not necessarily boost the cytosolic Ca2+ clearing.

In summary, the study of both mitochondrial and cytosolic Ca2+ dynamics would be fundamental in plants simultaneously lacking MCU isoforms and GLR3.5 (possibly with an inducible system) to define the role of mitochondria in clearing of cytosolic Ca2+ and therefore their role for the regulation of Ca2+ signalling. In addition to MCUs and GLR3.5, plant mitochondria may have other routes for Ca2+ uptake. Three-mitochondrial adenine nucleotide/phosphate carriers (AtAPC1–AtAPC3) 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 Ca2+ dynamics in the mitochondria and cytosol of apc mutants carrying mitochondria- and cytosol-targeted Ca2+ probes. Pollen tubes or root hairs, where both Ca2+ and ATP are fundamental players for a proper growth (Winship et al., 2016), might represent especially useful systems for these studies. Indeed, a recent study highlighted the importance of 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 Ca2+ 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 Ca2+ dynamics in the mitochondria and cytosol. The muc1, muc2, muc3, and glr3.5 plants showed mild phenotypes such as altered mitochondrial morphology, reduced pollen tube germination and growth in vitro, accelerated senescence, or reduced seedling root lengths that can be somewhat difficult to correlate directly with short-term signalling events. The lack of a strong phenotype in terms of mitochondrial and cytosolic Ca2+ dynamics and Ca2+-related signalling events can be explained by the lack of a true null mutant (unable to accumulate Ca2+ 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 Ca2+ in mitochondria is higher in the tip of the root hairs (500 nM) than in the shanks (200 nM), hence essentially following the cytosolic Ca2+ gradient (Wang et al., 2010). As mentioned above, growing pollen tubes and root hairs, that both have a high demand of metabolic energy and require the establishment of a defined cytosolic tip Ca2+ gradient, may represent the most suitable systems (Michard et al., 2017). Indeed, the recent demonstration that the muc2 mutant shows a phenotype in pollen tubes supports this idea (Selles et al., 2018). It might also be interesting to understand if and how mitochondrial Ca2+ release can regulate cytosolic Ca2+ recovery. In a simplified way, we may hypothesize that the slow decrease of mitochondrial Ca2+ might delay the cytosolic Ca2+ 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 Ca2+ from the mitochondria (Shao et al., 2016; Austin et al., 2017). Both Arabidopsis homologues, 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 Ca2+ 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 analyse the cytosol/mitochondria Ca2+ 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 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, Marchantia polymorpha, or 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 et al. (2013) who screened an A. 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 a negative regulator of salt and osmotic stress tolerance in Arabidopsis (Vanderauwera et al., 2012). Importantly, the authors revealed that 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 crossroads of ER stress and general cellular responses. A detailed description of Ca^{2+} dynamics in mitochondria, ER, and cytosol has not been provided, making it difficult to assign a specific role for mitochondria in the regulation of cytosolic Ca^{2+} under these experimental conditions. In summary, a suggested role for 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 also have to be taken into account, even if only a few studies addressed their involvement during the last few years. Peroxisomes are ubiquitous single-membrane–bounded organelles that fulfil essential roles in cellular metabolism. In contrast to mitochondria, peroxisomes do not have any ETC and, to the best of our knowledge, the existence of a membrane potential has not been reported. However, the peroxisomal membrane is impermeable to high molecular weight molecules (>1000 Da), and specific carriers are expressed in the organelles for the transport of different metabolites (Linka and Weber, 2010; Linka and Esser, 2012). Both mammalian and plant peroxisomes accumulate Ca^{2+} in the lumen in response to stimuli that trigger cytosolic Ca^{2+} increases (Lasorsa et al., 2008; Costa et al., 2010, 2013). The resting intraperoxisomal 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 a H^{+} gradient (Drago et al., 2008). In plant cells, only two reports showed a stimulus–induced peroxisomal Ca^{2+} increase in guard cells and root tip cells (Costa et al., 2010; 2013). In both cases, the peroxisomal Ca^{2+} dynamics were like the cytosolic dynamics, reminiscent of 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, catalase 3 (CAT3) controls the H_{2}O_{2} levels in guard cells (Zou et al., 2015), and this regulation is dependent on Ca^{2+} in two different ways—one mediated by CaM (Yang and Poovaliah, 2002) that operates in peroxisomes, and one mediated by CPK8 operating in the cytosol (Zou 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 photosynthetic enzymes, as well as for nitric oxide production (Corpas and Barroso, 2017). In conclusion, the property of peroxisomes to accumulate and release Ca^{2+} into and out of the lumen has a functional role in the 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 mM 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 pieces of evidence which demonstrate that the apoplast represents the primary source for the entry of Ca^{2+} into the cell upon the perception of a given stimulus. In fact, by chelating extracellular Ca^{2+}, using EGTA or BAPTA, or by blocking the PM non-selective cation channels with La^{3+} or Gd^{3+}, stimuli-induced cytosolic Ca^{2+} increases are strongly reduced if not completely abolished (Knight et al., 1996; Lamotte et al., 2004; Ali et al., 2007; Navazio et al., 2007). Despite the importance of the apoplast in the generation of cytosolic Ca^{2+} increases, a limited number of studies 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 dynamics. Remarkably, the authors also showed that the permanent washout of apoplastic Ca\(^{2+}\) determined a continuing aequorin signal decay, hence confirming the probe 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 \textit{cngc2} and \textit{cax1/cax3} mutants overaccumulate apoplastic Ca\(^{2+}\) compared with the wild type, when grown in the presence of high external Ca\(^{2+}\) in the medium (Wang et al., 2017). Interestingly, the overaccumulation of apoplastic Ca\(^{2+}\) in the \textit{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-localized Ca\(^{2+}\)/H\(^{+}\) exchangers makes this latter observation of primary importance since it supports the existence of a potential communication between the 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 programmes of plants. Genetic approaches using mutant plants defective in specific Ca²⁺ transporters/channels, together with pharmacological approaches using Ca²⁺ chelators and/or inhibitors of Ca²⁺ channels differentially distributed across cellular membranes, have elucidated how the different stimulus-specific cytosolic Ca²⁺ signatures often derive from the joint contribution of more than one source of Ca²⁺. Figure 2 summarizes the different channels/transporters possibly involved in Ca²⁺ fluxes in different intracellular membranes. Crossover among cellular compartments, possibly due to structurally close contacts, may also affect the ensuing global cytoplasmic Ca²⁺ 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 Ca²⁺ signalling in organelles, together with newly generated detection systems for measuring organelar Ca²⁺ concentrations in intact plants, should provide fruitful grounds for ground-breaking discoveries. The view is emerging that, beside transport modules and decoders that might play a role in shaping plant Ca²⁺ homeostasis within the plant cell. One of the greatest challenges in the field is the elucidation of how influx and efflux Ca²⁺ transporters/channels are regulated in a concerted manner to translate specific information into a Ca²⁺ signature.

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