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Plant organellar calcium signalling: an emerging field

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Abstract

This review provides a comprehensive overview of the established and emerging roles that organelles play in calcium signalling. The function of calcium as a secondary messenger in signal transduction networks is well documented in all eukaryotic organisms, but so far existing reviews have hardly addressed the role of organelles in calcium signalling, except for the nucleus. Therefore, a brief overview on the main calcium stores in plants—the vacuole, the endoplasmic reticulum, and the apoplast—is provided and knowledge on the regulation of calcium concentrations in different cellular compartments is summarized. The main focus of the review will be the calcium handling properties of chloroplasts, mitochondria, and peroxisomes. Recently, it became clear that these organelles not only undergo calcium regulation themselves, but are able to influence the Ca^{2+} signalling pathways of the cytoplasm and the entire cell. Furthermore, the relevance of recent discoveries in the animal field for the regulation of organellar calcium signals will be discussed and conclusions will be drawn regarding potential homologous mechanisms in plant cells. Finally, a short overview on bacterial calcium signalling is included to provide some ideas on the question where this typically eukaryotic signalling mechanism could have originated from during evolution.

Keywords

Bacteria; calcium flux; calcium signalling; CAS; chloroplast; EF-hand protein; ER; mitochondria; peroxisome

Introduction

Plants react to changing environmental conditions through immediate signal transduction pathways. One integral part of many signal transduction pathways, in both plant and animal cells, is the usage of free calcium ions (Ca^{2+}) as secondary messengers. As Ca^{2+} forms insoluble precipitates with phosphate, which would interfere with phosphate-based metabolism, cells actively translocate Ca^{2+} from their cytoplasm to organelles and extracellular compartments. This principle has been conserved throughout evolution from bacteria to eukaryotes. The resulting ~10 000-fold difference between cytoplasmic (~100

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nM) and non-cytoplasmic (mM) Ca^{2+} concentrations enables the generation of calcium signals by fast changes of cytoplasmic Ca^{2+} levels via membranelocalized Ca^{2+}-permeable channels. A wide variety of signals, including abiotic, biotic, and developmental stimuli, were observed to evoke specific spatiotemporal calcium transients which are further transduced by Ca^{2+} sensor proteins into a transcriptional and metabolic response. So far in plants, most of the research on Ca^{2+} signalling has been focused on the transport mechanisms for Ca^{2+} into and out of the cytoplasm as well as the components involved in decoding of cytoplasmic Ca^{2+} signals, which has been extensively reviewed (Clapham, 2007; McAinsh and Pittman, 2009;DeFalco *et al.*, 2010; Dodd *et al.*, 2010; Kudla *et al.*, 2010). However, recent advances demonstrate how different organelles are involved in the process of Ca^{2+} signalling. Therefore, this review starts with a brief summary of the role of the main Ca^{2+} storage compartments [the vacuole, endoplasmic reticulum (ER) and apoplast] and the main focus is on the emerging role of Ca^{2+} signalling processes in plastids, mitochondria, and peroxisomes. Finally, considering the endosymbiotic origin of chloroplasts and mitochondria, the review will end with a glimpse at bacterial Ca^{2+} signalling in order to propose some ideas regarding the evolutionary background of Ca^{2+} signalling processes in plants.

Main plant calcium stores: the vacuole, the ER, and the apoplast

The vacuole

The main storage compartment of calcium in plants is the central vacuole. Consequently, plants become hypersensitive to Ca^{2+} if vacuolar uptake is hampered (Cheng *et al.*, 2005). In dicotyledonous plants Ca^{2+} is preferentially stored in the leaf mesophyll rather than in the epidermal vacuole (Storey and Leigh, 2004; Conn *et al.*, 2011). Estimates of the free vacuolar Ca^{2+} concentration range from 0.2 mM to 1–5 mM and can reach a maximum of 80 mM of total Ca^{2+} (free and bound Ca^{2+} combined, Fig. 1) (Conn and Gilliham, 2010). Most of the Ca^{2+} is tightly bound to chelating agents, such as malate, citrate, and isocitrate, and, therefore, is not readily available for calcium signalling. Calcium ions might also be transiently bound to proteins, in a fashion comparable with the classical Ca^{2+}-binding storage proteins of the ER (e.g. calreticulin). For example, a radish vacuolar Ca^{2+}-binding protein (RVCaB) was found to improve the calcium storage capacity of the vacuole (Yuasa and Maeshima, 2001), whereas similar proteins in *Arabidopsis* were only found associated with the plasma membrane and probably do not play a role in calcium storage (Kato *et al.*, 2010). Interestingly, evidence is emerging that calcium is not only stored in the vacuole, but plays an important role in signalling there as well, mainly by influencing the activity of tonoplast (vacuolar membrane) localized ion transporters (Peiter, 2011). Various Ca^{2+} channels and transporters were identified in the tonoplast (Pottosin and Schonknecht, 2007). In the case of ligand-gated channels, Ca^{2+} fluxes were measured upon the addition of the signalling molecules inositol 1,4,5-triphosphate ($InsP_3$) and cyclic ADP-ribose (cADPR) (Allen *et al.*, 1995), although this could not be repeated in a more recent study (Pottosin *et al.*, 2009). Voltage-dependent Ca^{2+} channels and ligand-gated Ca^{2+} channels release Ca^{2+} into the cytoplasm and have been characterized mainly during the 1990s through electrophysiological studies (Sanders *et al.*, 2002). Although they have been well characterized for almost 20 years now, no molecular identity has been found for these channels so far, except for TPC1. AtTPC1, which has no other homologues in *Arabidopsis*, was confirmed to be the slow vacuolar (SV, named after its voltage-gated characteristics) channel of the tonoplast (Hedrich and Neher, 1987; Peiter *et al.*, 2005). Despite being the major vacuolar calcium release channel (Pottosin *et al.*, 2009), debate surrounds the physiological contribution of the SV channel to calcium signalling, since knockout or overexpression of AtTPC1 has no effect on the calcium signals of a whole range of biotic and abiotic stresses (Ranf *et al.*, 2008). Moreover, the robust phenotype of *tpc1* mutants—

impaired stomatal closure upon extracellular Ca^{2+} elevation—is not due to impaired Ca^{2+} homeostasis in guard cells (Peiter *et al.*, 2005; Islam *et al.*, 2010), and evidence for a physiologically relevant release of Ca^{2+} from the vacuole still needs to be provided (Hedrich and Marten, 2011).

The ER

The calcium storage role of the ER is probably best known from human and animals, where the molecular mechanisms of calcium release from and uptake by the sarcoplasmic endoreticulum (SR; the ER of the muscle cell) during muscle contractions have been described in detail (Rossi and Dirksen, 2006). In animals, the total Ca^{2+} concentration in the ER is estimated to be 2 mM, while the free Ca^{2+} concentration varies between 50 μM and 500 μM (Fig. 1) (Coe and Michalak, 2009). In contrast, in plants, few data are available that describe the calcium storage properties of the ER. Research is hampered mainly due to the absence of direct homologues of the well-characterized mammalian $InsP_3$ and ryanodine receptors that are responsible for ER Ca^{2+} efflux in higher plant genomes. Interestingly though, the genomes of several algae species, including *Volvox* and *Chlamydomonas*, do contain these receptor proteins, suggesting they were present in ancestral eukaryotes and were lost by land plants after their divergence from the chlorophyte algae (Wheeler and Brownlee, 2008). Although higher plant genomes lack these canonical receptors, calcium fluxes have been measured from ER membrane preparations upon stimulation with $InsP_3$ and cADPR (Muir and Sanders, 1997; Navazio *et al.*, 2001). Moreover, the signalling molecule nicotinic acid adenine dinucleotide phosphate (NAADP), that was originally described to evoke calcium release from the ER in marine invertebrate species, is active in higher plants as well (Navazio *et al.*, 2000). Voltage-gated Ca^{2+} fluxes were also measured in ER membrane preparations of the tactile organs of *Bryonia dioica* (Klusener *et al.*, 1995; Klusener *et al.*, 1997) and in root-tip endomembranes of *Lepidium sativum* L. (Klusener and Weiler, 1999). However, whether this is a more widespread phenomenon in the ER of higher plants still needs to be established. Calreticulin, a luminal Ca^{2+}-binding protein of the ER, was found to be important for Ca^{2+} homeostasis in plants, but also serves as a chaperone for protein folding (Christensen *et al.*, 2010). The overexpression of maize calreticulin in tobacco cells lead to increased Ca^{2+} retention in the ER, and the down-regulation of calreticulin expression in *Arabidopsis* resulted in enhanced sensitivity of the plants to low Ca^{2+} (Persson *et al.*, 2001). A comparison of Ca^{2+}-binding proteins between animals and plants suggests that calreticulin is the major Ca^{2+} storage protein in plants (Nagata *et al.*, 2004). In conclusion, little is known about plant ER Ca^{2+} storage and release compared with the animal field, which is reflected by the fact that the molecular identity of the plant ER Ca^{2+} release channel(s) is still unknown to date.

The apoplast

The apoplast is another major plant Ca^{2+} store. At the same time it acts as the ‘highway’ through which Ca^{2+} is trafficked to the cells by means of the water transpiration stream. Furthermore, the Ca^{2+} concentration in the apoplast needs to be tightly regulated as a high apoplastic Ca^{2+} concentration impairs stomatal movement (Kim *et al.*, 2010) and plant cell wall rigidity depends on Ca^{2+} for pectate cross-linking (Hepler, 2005). That these two reasons can drastically affect plant growth was recently demonstrated by the analysis of a mutant plant that is deficient in the main vacuolar Ca^{2+} importers AtCAX1 and AtCAX3 (Conn *et al.*, 2011). The authors reasoned that deficient Ca^{2+} sequestration in the vacuole led to an increase in free Ca^{2+} in the apoplast, with the above-mentioned defects as a consequence. Most of the Ca^{2+} in the apoplast is bound to negatively charged carboxyl groups of pectin and oxalates, and reports on the concentration of Ca^{2+} vary from 10 μM to 10 mM (Hepler, 2005). Conn and colleagues (2011) estimated the free Ca^{2+} concentration in the *Arabidopsis* leaf apoplast to be ~0.33 mM, and the concentration of bound Ca^{2+} to be 0.5

mM (Fig. 1). In addition to the voltage-gated Ca^{2+} channels, other ligand-gated ion channels, that can mediate fluxes of Ca^{2+} into the cytoplasm, include the cyclic nucleotide-gated channels (CNGCs) and glutamate receptor-like channels (GLRs). Both gene families in *Arabidopsis* contain 20 members and they can carry a diversity of ions, but some members have been implicated in mediating cytosolic increases of Ca^{2+} (Ali *et al.*, 2006, 2007; Hua *et al.*, 2003; Qi *et al.*, 2006; Kaplan *et al.*, 2007). All CNGCs and GLRs studied so far are targeted to the plasma membrane.

In conclusion, it should be noted that being large calcium stores does not automatically mean that the apoplast and the vacuole are the only relevant organelles for calcium signalling in plants. The vacuole and apopoplast, but also the ER, each contain an equally high potential for unloading Ca^{2+} into the cytoplasm. Furthermore, the sequestering of such high amounts of Ca^{2+} plays a role not only in signalling, but also in the general ion homeostasis of the plant. Also the types and amounts of channels that are present at the respective subcellular membranes to generate specific Ca^{2+} fluxes should be considered. This is exemplified by the SV channel AtTPC1, which is the main vacuolar Ca^{2+} release channel, but is probably more involved in general ion homeostasis than in signalling to biotic or abiotic stresses. On a related note, calcium release channels from the above-mentioned subcellular calcium stores were found to influence specific Ca^{2+} fluxes (reviewed in McAinsh and Pittman, 2009). This reflects the heterogeneous nature of calcium signalling in which various organelles can be responsible for calcium uptake and release from the cytosol. Therefore, to obtain a broader, more comprehensive understanding of calcium signalling, the role of other organelles such as chloroplasts and mitochondria should be considered.

Organellar calcium signalling

Chloroplast calcium signalling

Already some decades ago it was found that Ca^{2+} modulates the metabolic reactions of the chloroplast. Elevated Ca^{2+} concentrations effectively inhibit the Calvin–Benson cycle enzymes fructose-1,6-bisphophatase and sedoheptulose bisphosphatase, leading to a halt of photosynthetic CO_2 fixation (Racker and Schroeder, 1958; Portis and Heldt, 1976; Charles and Halliwell, 1980). The total concentration of Ca^{2+} in the chloroplast has been estimated at 15 mM or higher (Nobel, 1969; Portis and Heldt, 1976) and increases upon illumination, by the uptake of Ca^{2+} from the external medium during the daylight (Kreimer *et al.*, 1985; Roh *et al.*, 1998). Because a high amount of free chloroplastic Ca^{2+} would inhibit photosynthesis and precipitate abundant chloroplastic phosphate, most of the Ca^{2+} is bound to thylakoid membranes or to stromal proteins (Gross and Hess, 1974; Davis and Gross, 1975; Kreimer *et al.*, 1987). Accordingly, the resting free Ca^{2+} concentration in the stroma during the day was estimated to be ~150 nM (Fig. 1) (Johnson *et al.*, 1995). Calcium also affects the photosynthetic reactions from the luminal side of the thylakoid. It is an essential cofactor of the oxygen-evolving complex and binds the 8 kDa subunit of the ATP synthase, thereby regulating the photosynthetic proton flow and ATP production (Zakharov *et al.*, 1993; Ifuku *et al.*, 2010). So, the chloroplast has an essential requirement for Ca^{2+}, but needs tight control over its distribution, as indicated in Fig. 2.

Using chloroplast-targeted Aequorin reporter lines, calcium fluxes were detected in chloroplasts in daily rhythms after the transition from light to dark (Johnson *et al.*, 1995; Sai and Johnson, 2002). This stromal Ca^{2+} flux after the light–dark transition is proposed to be responsible for inhibiting photosynthetic CO_2 fixation during the night and could help to entrain the circadian clock. The characteristics of these Ca^{2+} fluxes suggest that chloroplasts take up Ca^{2+} from the cytosol during the light period and store it in the thylakoid membrane or in another as yet unknown store. Upon transition from light to dark the Ca^{2+} is

subsequently released from the store back into the cytosol. Light, via the thylakoid proton gradient, seems to drive Ca^{2+} uptake into the thylakoid lumen as well, through the activity of a Ca^{2+}/H^{+} exchanger (Ettinger *et al.*, 1999). However, the inhibition of the photosynthetic electron transport chain, and correspondingly the proton gradient, resulted in a slight increase of stromal Ca^{2+} during the light period, but did not inhibit the charging of the Ca^{2+} store that was discharged by lights off (Sai and Johnson, 2002). Hence, the authors proposed the existence of an unknown alternative stromal Ca^{2+} store.

The molecular nature of the chloroplast Ca^{2+} store is a long-standing question, and the massive difference between the total and free resting Ca^{2+} concentration (Fig.1) calls for a strong Ca^{2+}-buffering mechanism in the chloroplast. It has long been known that the net negative charge of the thylakoid surface, which has been referred to as ‘a diffuse electrical layer’ (Barber *et al.*, 1977), is balanced by the positive charge of magnesium ions (Mg^{2+}) (Nakatani *et al.*, 1979; Jennings *et al.*, 1981). Recently, Fristedt and colleagues reported that the negative charge is mainly due to the gross phosphorylation of the photosynthetic complexes and that mutation of the involved kinases, STN7 and STN8 (State Transition 7 and 8), influences the Mg^{2+}-dependent stacking of the thylakoid (Fristedt *et al.*, 2009, 2010). Furthermore, the STN7 and STN8 kinases regulate phosphorylation of the photosynthetic complexes in a light-dependent manner (Depege *et al.*, 2003). Since Ca^{2+} has highly similar metal properties to Mg^{2+}, it is feasible that phosphorylated protein residues of the thylakoid account for much of the Ca^{2+}-buffering capacity of the chloroplast. Considering Ca^{2+} transients on light to dark transitions, the link with thylakoid protein phosphorylation seems an interesting route for further investigation. Furthermore, the influence of pH should also be considered, as pH is known to influence the binding capacity of Ca^{2+} storage proteins (Hidalgo *et al.*, 1996), due to protonation of the acidic residues involved in Ca^{2+} binding. Particularly in chloroplasts, where dramatic differences in pH are present during light conditions and change in the transition from light to dark, this is expected to impact on calcium signalling significantly.

Active Ca^{2+} transport has also been measured across the chloroplast inner envelope and could account for the observed Ca^{2+} uptake of the chloroplast during the light period. A negative inside membrane potential-driven Ca^{2+} transport (uniport) was measured from inner envelope membrane vesicles of *Pisum sativum*, confirming previous experiments with intact chloroplasts (Kreimer *et al.*, 1985;Roh *et al.*, 1998). This observation is strengthened by measurements with a calcium ion-selective microelectrode in intact algal cells that recorded a photosynthesis-dependent decrease in cytoplasmic Ca^{2+} levels (Miller and Sanders, 1987). However, the molecular identity of this channel has not been described. On the other hand, two potential Ca^{2+} ATPases were identified in the chloroplast envelope. The first is AtACA1 from the autoinhibited Ca^{2+}-ATPases family and is most probably found only in root plastids (Huang *et al.*, 1993). Strangely though, the laboratories of both Huang and Roh could not find Ca^{2+}-ATPase activity at the envelope. Furthermore, since its description in 1993, ACA1 has been found in cauliflower tonoplast and *Arabidopsis* ER, prompting further study on this Ca^{2+}-ATPase (Malmstrom *et al.*, 1997; Dunkley *et al.*, 2006). The second Ca^{2+}-ATPase might be AtHMA1, a member of the heavy metal P-type ATPases that was shown to have high-affinity Ca^{2+} transport activity and is specifically inhibited by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (Moreno *et al.*, 2008). There is dispute about its exact role, because originally it was described to transport Cu^{2+} into the chloroplast (Seigneurin-Berny *et al.*, 2006) but later on it was shown to function in the tolerance to excess Zn^{2+}, by extruding it from the chloroplast (Kim *et al.*, 2009). ALBINO3 (ALB3), the integral membrane protein and translocase involved in chloroplast biogenesis (Sundberg *et al.*, 1997; Lewis *et al.*, 2010), might function as a Ca^{2+} transporter at the thylakoid. The pea homologue of ALB3, *Pisum*-post-floral-specific gene 1 (PPF1), produced significant inward calcium ion currents in Novikoff

human hepatoma cells, and the Ca^{2+} homeostasis of plants with altered expression of PPF1/ALB3 was disrupted in *Arabidopsis* guard cells, leading to severe growth phenotypes (Wang *et al.*, 2003; Li *et al.*, 2004). In conclusion, although several transporters are hypothesized to aid chloroplast Ca^{2+} influx, a more comprehensive analysis is needed to link these proteins directly to chloroplast Ca^{2+} homeostasis. Furthermore, how Ca^{2+} is exported from the thylakoid lumen to the stroma and subsequently to the cytosol remains largely open. To this end, Roh and colleagues showed that Ca^{2+} can traverse the inner envelope through a reversal of the membrane potential-driven Ca^{2+} transporter (Roh *et al.*, 1998), and interesting progress on mitochondrial Ca^{2+} transport in mammals could shed light on the underlying molecular basis of calcium transport across the chloroplast envelope (see mitochondrial calcium signalling).

From more recent work, it became apparent that calmodulin (CaM) has regulatory roles in chloroplasts. The best example is the import of nuclear-encoded chloroplast proteins via the TOC (translocon at the outer envelope of chloroplasts) and TIC (translocon at the inner envelope of chloroplasts) complexes. Calcium/CaM was found to promote chloroplast import, and this is most likely to be due to the direct interaction of calmodulin with the stromal side of Tic32 (TIC protein of 32 kDa) (Chigri *et al.*, 2005, 2006). Furthermore, in a different study, electrophysiological measurements revealed that the gating properties of the main pore-forming subunit of the TIC complex, Tic110, were affected in a specific manner by Ca^{2+} (Balsera *et al.*, 2009). Taken together, Ca^{2+} seems to influence chloroplast protein import at these closely linked sites. Another function of the chloroplast that was found to be modulated by Ca^{2+} is the chloroplast inner vesicle transport system (Morre *et al.*, 1991). This system is proposed to have a role in thylakoid membrane biogenesis and was found to be disrupted by CaM inhibitors as well as calcium depletion (Westphal *et al.*, 2001, 2003). Chloroplast division might also be regulated in a Ca^{2+}-dependent manner by AtMinD1 (*Arabidopsis* Minicell D1), which is a part of the chloroplast division machinery, because its ATPase activity depends on Ca^{2+}, rather than Mg^{2+} as is the case in bacteria (Aldridge and Moller, 2005). Furthermore, two chloroplastic mechanosensitive ion channels MSL2 and 3 [homologues of the bacterial mechanosensitive (MS) channel MscS: MscS-Like 2 and 3] were found to influence chloroplast division and act in concert with the Min proteins (Haswell and Meyerowitz, 2006; Wilson *et al.*, 2011). It would be interesting to see if MSL2 and 3 can directly influence the Ca^{2+}-dependent activity of AtMinD1, either by mediating Ca^{2+} fluxes or through a depolarization of the chloroplast envelope.

Chloroplast movement in response to fluctuating light conditions is another Ca^{2+}-dependent process shown to occur in different species, such as *Lemna trisulca* and tobacco (Tlalka and Fricker, 1999; Anielska-Mazur *et al.*, 2009). Chloroplasts move along the actin cytoskeleton in plant cells (Kong and Wada, 2011), and accordingly the use of Ca^{2+} chelators and CaM inhibitors revealed the stabilizing effect of Ca^{2+} on actin polymerization and its importance for chloroplast movement. However, chloroplast movement was influenced even in the presence of an intact actin network, thereby evoking a signalling function of Ca^{2+} in light-induced chloroplast movements.

NAD kinase (NADK) is an intriguing case for chloroplastic calcium-dependent regulations. Through the use of CaM inhibitors, NADK was the first plant enzyme found to be activated by CaM, and the majority of its activity was found to localize to the chloroplast (Jarrett *et al.*, 1982). Jarrett and colleagues purified a CaM-containing fraction from pea chloroplasts to near homogeneity, but did not identify the protein responsible for NADK activation. The initial increase of Ca^{2+} in the chloroplast upon illumination was proposed to activate NADK via the interaction with CaM. NADK catalyses the light-dependent conversion of NAD to NADP, the final electron acceptor of the photosynthetic electron transport chain. Thereby it provides an important reduction potential for the plant, which becomes obvious in the

reduced growth and hypersensitivity to oxidative stress of chloroplastic NADK knock-out plants (Chai *et al.*, 2005; Takahashi *et al.*, 2006). From more recent work, it became apparent that one of the three *Arabidopsis* NADK isoforms, AtNADK2, is responsible for the CaM-dependent NADK activity in chloroplasts (Turner *et al.*, 2004; Waller *et al.*, 2010). A region of 45 amino acids within the long N-terminal extension of AtNADK2 was found to be sufficient and necessary to bind to CaM. Other examples of chloroplast proteins that were found to interact with CaM are AtPsaN (a subunit of photosystem I), the chaperonin AtCPN10, and AtAFG1L1, an AAA^+-ATPase (Yang and Poovaiah, 2000; Reddy *et al.*, 2002; Bussemer *et al.*, 2009). However, the functional relevance of these interactions needs further study. To conclude, although a chloroplastic CaM or CaM-like protein is still unknown, various stromal proteins are able to bind to CaM, leading to a change of their activity. In the same context, a Ca^{2+}-dependent protein kinase activity and cross-talk between Ca^{2+} signalling and protein phosphorylation is described in two accompanying papers in this issue (Bayer *et al.*, 2012;Stael *et al.*, 2012). Therefore, the identification of CaMs or other Ca^{2+}-binding proteins in chloroplasts is expected greatly to advance the understanding of the physiological relevance of these calcium/CaM-dependent regulations.

So far there are two reports of EF-hand-containing proteins in the chloroplast. The best documented is the Ca^{2+}-activated RelA/SpoT homologous protein (CRSH), that is novel both for being a chloroplastic EF-hand protein and for its alleged function. CRSH contains two EF-hands and a RelA/SpoT enzymatic domain, which is responsible for the calcium-dependent production of a small signalling nucleotide, guanosine 5′-diphosphate 3′-diphosphate (ppGpp) (Masuda *et al.*, 2008). ppGpp signalling was discovered in bacteria as a response to stress conditions, such as nutrient deprivation, in a process called ‘the bacterial stringent response’, and homologous proteins have since been found in various plant species (Tozawa *et al.*, 2007; Masuda *et al.*, 2008). The bacterial RelA and SpoT proteins do not contain EF-hands, which seems to be an exclusive trait of CRSH in higher plants. ppGpp was found mainly in the chloroplast, and the levels changed after different stress and hormonal treatments and upon the transition from light to dark (Takahashi *et al.*, 2004). Similar to its function in transcription and translation in bacteria, ppGpp was found to modulate exclusively the function of the bacterial type plastid-encoded plastid RNA polymerase (PEP), but not the nuclear-encoded plastid RNA polymerase (NEP) (Sato *et al.*, 2009). It seems that the bacterial stringent response has been conserved in chloroplasts from its cyanobacterial origin, but more experimental work is needed to elucidate its physiological function and the interplay with Ca^{2+} signalling, in plants. The second chloroplastic EF-hand protein is a substrate carrier (AtSAMTL, for SAMT-like) that contains a single EF-hand and belongs to the mitochondrial carrier protein family. It was recently found in a targeted proteomics screen and was confirmed to reside in the chloroplast envelope by yellow fluorescent protein (YFP) fusion analysis (Bayer *et al.*, 2011). Based on a detailed functional prediction, it is proposed to import *S*-adenosylmethionine (SAM) into the chloroplast in addition to the better described SAM transporter, SAMT (Stael *et al.*, 2011*b*).

Reports on a chloroplast-localized protein involved in ‘calcium sensing’ (AtCAS) evoke the idea that chloroplasts may modulate cytoplasmic Ca^{2+} signalling. AtCAS was first reported as a plasma membrane-localized Ca^{2+}-sensing receptor, important for inducing stomatal closure provoked by elevation of the extracellular Ca^{2+} concentration ($[Ca^{2+}]_{ext}$)—a hallmark of stomatal movement (Han *et al.*, 2003). The protein was found to bind Ca^{2+} with a low affinity and high capacity, and down-regulation of its expression impaired the production of $[Ca^{2+}]_{ext}$-induced cytoplasmic Ca^{2+} oscillations. However, subsequent reports identified AtCAS to be targeted to the thylakoid membrane (Friso *et al.*, 2004; Nomura *et al.*, 2008; Vainonen *et al.*, 2008; Weinl *et al.*, 2008). Nevertheless, knock-out of AtCAS was still confirmed, by different labs, to impair $[Ca^{2+}]_{ext}$-induced stomatal closure, whereas

overexpression of CAS promoted stomatal closure in the absence of externally applied Ca^{2+} (Nomura *et al.*, 2008; Weinl *et al.*, 2008). Disruption of AtCAS in *Arabidopsis* retarded plant growth, and AtCAS was found to be increasingly phosphorylated by the state transition kinase AtSTN8 under increasing light intensities (Vainonen *et al.*, 2008). Similarly, CAS is required for photoacclimation in the process of non-photochemical quenching (NPQ) in *Chlamydomonas* where *cas*-knockdown lines displayed a high-light-sensitive phenotype (Petroutsos *et al.*, 2011). Stomata of *cas Arabidopsis* plants displayed normal closure after the application of externally imposed cytoplasmic Ca^{2+} oscillations, indicating that the guard cells are still responsive to Ca^{2+} signals but most probably have a defect in the generation of Ca^{2+} fluxes (Weinl *et al.*, 2008). This suggests that the chloroplast can sense and influence cytoplasmic Ca^{2+} levels; however, the molecular mechanisms behind these processes still await discovery.

Mitochondrial calcium signalling

In contrast to the animal and human field, where mitochondrial calcium uptake and release are well studied and known to play important cellular roles, relatively few data are available on calcium signalling in plant mitochondria. It is now well established that mitochondria in animals function as transient calcium stores that can produce Ca^{2+} microdomains through a close interaction with the ER, thereby modulating Ca^{2+} signatures (Clapham, 2007; Laude and Simpson, 2009). On the other hand, the elevation of Ca^{2+} inside the mitochondria positively affects ATP production by up-regulating the major limiting enzymes of the citric acid cycle (similar to the up-regulation of the Calvin cycle in chloroplasts) (McCormack *et al.*, 1990). The overaccumulation of Ca^{2+} in the mitochondria is linked to the induction of apoptosis by opening of the mitochondrial permeability transition pore (mPTP) and the subsequent release of mitochondrial apoptosis markers, such as cytochrome *c* (Giacomello *et al.*, 2007; Szabadkai and Duchen, 2008). This process apparently occurs in plants as well (Arpagaus *et al.*, 2002; Tiwari *et al.*, 2002; Virolainen *et al.*, 2002).

In plants, the resting free Ca^{2+} concentration in the mitochondria was estimated to be ~200 nM (Logan and Knight, 2003), with most of the mitochondrial Ca^{2+} probably being bound in the form of a ready-releasable amorphous phosphate precipitate (Fig. 1) (Chalmers and Nicholls, 2003; Starkov, 2010). Ca^{2+} fluxes in *Arabidopsis* mitochondria have been observed upon various stimulations (Logan and Knight, 2003). Whereas most mitochondrial Ca^{2+} fluxes were similar, H_2O_2 and touch stimulation produced a signal that was different from the concurrently occurring cytosolic Ca^{2+} fluxes. This indicates that mitochondria are not just passive calcium sinks, but are able to regulate their own Ca^{2+} fluxes as depicted in Fig. 2.

Surprisingly little information about Ca^{2+} transporters of plant mitochondria exists, compared with the knowledge on this topic in the animal and human field (Collins and Meyer, 2010; Starkov, 2010). Animal mitochondria take up high or low concentrations of Ca^{2+} differently, and various Ca^{2+} channels have recently been described (Hajnoczky and Csordas, 2010; Hoppe, 2010). The mitochondrial calcium uniporter (MCU) imports Ca^{2+} from ‘microdomains’ with highly elevated cytosolic Ca^{2+} concentrations. MCU has been extensively studied and its properties are well delineated: (i) electrogenic transport that is driven by a negative inside membrane potential; (ii) sensitivity to ruthenium red; (iii) low affinity for Ca^{2+}; and (iv) regulation by Ca^{2+}. After almost 50 years, the molecular nature of the uniporter has been discovered in animals in a series of recent studies (Perocchi *et al.*, 2010; Baughman *et al.*, 2011; De Stefani *et al.*, 2011). In an integrative genomics and proteomics search, Perocchi and colleagues found an EF-hand-containing protein, which is targeted to the mitochondrial inner membrane, to induce a Ca^{2+}-dependent calcium influx into the mitochondria. They called it MICU1, for mitochondrial Ca^{2+} uptake 1, and reasoned

that it is most probably not the uniporter itself, but the sensor for Ca^{2+} that regulates the MCU. In two independent follow-up experiments, based on the phylogenetic profile, protein distribution, and characteristics of MICU1, a 40 kDa protein was identified as the actual Ca^{2+} pore and therefore it was called MCU. The MCU protein has two transmembrane helices, connected by a conserved loop containing the amino acids DIME, and most probably oligomerizes in the mitochondrial inner membrane. Intriguingly, the *Arabidopsis* genome contains six uncharacterized genes with a rather low homology to the human MCU, which share the pore-forming domain with two transmembrane helices connected by a conserved DVME motive (Fig. 3A). These potential *Arabidopsis* MCU isoforms are highly predicted to localize to the mitochondria, except for At5g66650, which is equally predicted to target to the chloroplast according to Aramemnon (Schwacke *et al.*, 2003) (Fig. 3B). A quick survey of the expression profiles by GENEVESTIGATOR (Zimmermann *et al.*, 2004) suggests a differential tissue expression of the various isoforms, for example to the flower, root, or guard cell. The possibility that an MCU isoform is located in the chloroplast is supported by the notion that the light-dependent Ca^{2+} influx in chloroplasts, reported by Kreimer *et al.* (1985), is inhibited by ruthenium red and that the overall characteristics are similar to the mitochondrial Ca^{2+} uniport. Moreover, the potential chloroplastic localization and preferential guard cell expression of At5g66650 are interesting in the light of stomatal movement, since chloroplasts have been implicated in ‘sensing’ extracellular calcium transients through CAS protein and the modulation of stomatal closure.

In addition to the low-affinity MCU system, mammalian mitochondria take up Ca^{2+} at lower cytoplasmic concentrations via the high-affinity Ca^{2+}/H^{+} antiporter, LETM1 (Jiang *et al.*, 2009). Interestingly, the closest *Arabidopsis* homologue of LETM1, the mitochondrial high-affinity Ca^{2+}/H^{+} antiporter, contains two EF-hands and was recently found to localize to the mitochondria by green fluorescent protein (GFP) fusion analysis (Van Aken *et al.*, 2009). In this study the authors attempted to delineate the mitochondrial stress response by searching for mitochondrial proteins that show the greatest expression variation upon 16 selected stress treatments. The fact that the AtLETM1-like protein was included in this set further prompts the study of this protein for its Ca^{2+}/H^{+} activity and the involvement of mitochondrial calcium signalling upon stress conditions in *Arabidopsis*. In mammals, calcium efflux from mitochondria can occur through a reversal of LETM1 or through the Na^{+}/Ca^{2+} exchanger NCLX, that was recently found to be targeted to the mitochondria (Palty *et al.*, 2010). To conclude, the presence of homologues in the *Arabidopsis* genome of all the above-mentioned proteins raises the interesting possibility of translating the mammalian findings directly into plants.

Calcium/CaM was reported to promote mitochondrial protein import in a manner similar to how it occurs in chloroplasts (Kuhn *et al.*, 2009). Furthermore, it was found to be a plant-specific trait, because yeast mitochondria were not susceptible to the CaM inhibitors and calcium ionophores used in the study. Pyruvate dehydrogenase activity was also found to decline with the use of CaM inhibitors (Miernyk *et al.*, 1987). The pyruvate dehydrogenase complex (PDC) assists in the conversion of pyruvate into acetyl-CoA and, therefore, an important connection between glycolysis and the citric acid cycle might be regulated by Ca^{2+} in plants. The finding of a CaM-binding AAA^{+}-ATPase, AtAFG1L1, with dual localization to chloroplasts and mitochondria further suggests the presence of CaM in the plant mitochondria (Bussemer *et al.*, 2009). So far, the only report on CaM in the mitochondria came from Biro and colleagues, who found an oat CaM in the intermembrane space that was lost upon removal of the outer mitochondrial membrane (Biro *et al.*, 1984).

The glutamate dehydrogenase β subunit (GD-β) localizes to the mitochondrial matrix, contains a single EF-hand, and its activity was reported to be stimulated by the addition of Ca^{2+} (Turano *et al.*, 1997). Two isoforms of the type II NAD(P)H:quinone oxidoreductases

of *Arabidopsis*, AtNDB1 and AtNDB2 were found to be externally attached to the inner membrane of mitochondria and to contain functional EF-hands (Geisler *et al.*, 2007).

Ample evidence for the influence of mitochondrial Ca^{2+} on cytoplasmic Ca^{2+} fluxes exists in animals (Laude and Simpson, 2009), and these observations probably hold true for plants, given that plant mitochondria exhibit a similar handling of Ca^{2+} (Silva *et al.*, 1992). Maize mitochondria have been shown to release Ca^{2+} upon hypoxia, and their internal Ca^{2+} concentration changes rapidly and in a reversible manner when returning back to normoxia (Subbaiah *et al.*, 1994, 1998). The recently described mitochondrial carrier proteins APC1, 2, and 3 (short for ATP-phosphate carrier1, 2, and 3) could be immediate targets of these mitochondrial Ca^{2+} fluxes (Stael *et al.*, 2011*b*). APC1–APC3 each contain four EF-hands and are better known from yeast and mammals to import ATP into mitochondria in a Ca^{2+}-dependent manner (Chen, 2004; Traba *et al.*, 2008). Mitochondrial ATP import might be a first line of defence against hypoxia, in order to maintain the mitochondrial membrane potential and cell viability by a reversal of the F_oF_1 H^+-ATP synthase. Other evidence for mitochondrial Ca^{2+} efflux comes from the measurement of Ca^{2+} changes in the root hair in response to the disruption of actin polymerization by latrunculin B (Lat-B) and Jasplakinolide (Jas) (Wang *et al.*, 2010). Root hair cells produce a tip-focused Ca^{2+} gradient, with Ca^{2+} oscillation lagging behind growth oscillation, in a similar fashion to growing pollen tubes (Cardenas *et al.*, 2008; Monshausen *et al.*, 2008). Modulation of actin polymerization caused a release of Ca^{2+} from the mitochondria, probably via the mPTP, and a concurrent increase in cytosolic Ca^{2+} concentrations. Furthermore, Wang and colleagues showed that the concentration of free Ca^{2+} in mitochondria displays a gradient from a high concentration at the growing tip (~500 nM) to low at the base (~200 nM). Together with the observation that mitochondria shuttle up and down the actin filaments through the root hair (Zheng *et al.*, 2009), mitochondria may play a role in the removal or buffering of the Ca^{2+} fluxes in the root hair apex, as was previously hypothesized (Hetherington and Brownlee, 2004).

Peroxisomal calcium signalling

Information on calcium signalling in the peroxisome is scarce. It was only recently that the occurrence of calcium fluxes in the peroxisome was recognized in mammals (Raychaudhury *et al.*, 2006; Drago *et al.*, 2008; Lasorsa *et al.*, 2008). Two different resting free Ca^{2+} concentrations have been reported from these experiments, namely 150 nM (Drago *et al.*, 2008) and 2 μM (Lasorsa *et al.*, 2008). A similar study, based on the expression of a peroxisomal-targeted chameleon probe (a Ca^{2+} reporter protein construct), showed that plant peroxisomes undergo Ca^{2+} fluxes as well (Costa *et al.*, 2010). Furthermore, the peroxisomal Ca^{2+} increase was found to enhance the detoxification of the reactive oxygen species (ROS) H_2O_2 *in vivo* through the activity of the catalase isoform 3 (AtCAT3), as was proposed earlier. In 2002, Yang and Poovaiah demonstrated the *in vitro* stimulation of AtCAT3 activity by the calcium-dependent binding of CaM, and provided evidence for the presence of CaM in peroxisomes. However, they did not find its molecular identity (Yang and Poovaiah, 2002). A member of the *Arabidopsis* calcium-dependent protein kinase family, AtCPK1, was found to bind to the external surface of peroxisomes (Dammann *et al.*, 2003; Coca and San Segundo, 2010) and lipid bodies, which is most likely to be due to an N-terminal myristoylation signal, which determines the localization of many calcium-dependent protein kinases (CDPKs) and other kinases (Lu and Hrabak, 2002; Benetka *et al.*, 2008; Stael *et al.*, 2011*a*). AtCPK1 was shown to mediate pathogen resistance (Coca and San Segundo, 2010). Given the few reports, the already presented interplay between calcium and ROS prompts the further study of the molecular nature of calcium handling and signalling in and around peroxisomes.

Nuclear calcium signalling

Nuclear Ca^{2+} in plants has recently been reviewed comprehensively (Mazars *et al.*, 2009, 2011) and will therefore be discussed only briefly. Calcium signals in the nucleus enable the cell to react to environmental changes by alteration of gene expression in animals and plants (Ikura *et al.*, 2002; M.C. Kim *et al.*, 2009; Galon *et al.*, 2010;Reddy *et al.*, 2011). This may sound straightforward; however, only recently were direct target genes of Ca^{2+}-dependent gene expression reported in *Arabidopsis* (Kaplan *et al.*, 2006). The main problem is to distinguish them from non-Ca^{2+}-dependent gene expression mediated via different nuclear signalling pathways in response to stress treatment (Finkler *et al.*, 2007; Wurzinger *et al.*, 2011). Various studies revealed that many of the Ca^{2+}-regulated genes contained abscisic acid-responsive element (ABRE)-related *cis*-elements and were already implicated in the abiotic stress response before (Hirayama and Shinozaki, 2010). Ca^{2+} can influence transcription through Ca^{2+}-binding transcription factors, CaM-binding transcription activators [AtCAMTAs, six members in *Arabidopsis* (Bouche *et al.*, 2002)], or phosphorylation of transcription factors by CDPKs, of which quite a number are present in the nucleus (Dammann *et al.*, 2003;Choi *et al.*, 2005; Zhu *et al.*, 2007; Boudsocq *et al.*, 2010;Mehlmer *et al.*, 2010).

Various stimuli such as abiotic or biotic stresses as well as symbiotic signals elicit nuclear Ca^{2+} fluxes (Pauly *et al.*, 2000;Lecourieux *et al.*, 2005; Oldroyd and Downie, 2006; Sieberer *et al.*, 2009). A lot of the research on nuclear Ca^{2+} signalling has been focused on the question whether nuclei can generate Ca^{2+} fluxes autonomously from the cytosol. The nuclear resting free Ca^{2+} concentration was measured at ~100 nM (Brini *et al.*, 1993; Mazars *et al.*, 2009), which is similar to the cytosolic value (Fig. 1). The fact that the nucleoplasm and cytosol are connected by relatively large pores in the nuclear envelope might suggest that nuclear Ca^{2+} fluxes are the result of passive influx from the cytosol. However, the delays that have been measured between cytosolic and nuclear Ca^{2+} fluxes implicate the opposite. Furthermore, when tobacco cells were treated with a biologically active derivate of jasmonate (jasmonate-isoleucine), nuclei were able to generate Ca^{2+} fluxes without any measurable cytosolic Ca^{2+} responses (Walter *et al.*, 2007). Experiments with isolated nuclei further emphasized the autonomous nature of plant nuclei from the extranuclear environment with regards to Ca^{2+} signalling (Xiong *et al.*, 2004, 2008).

If the nucleus is able to produce its own Ca^{2+} fluxes, than the nuclear envelope is most likely to serve as the responsible Ca^{2+} store. Analysis of its protein components has been hampered by the difficult extraction of intact nuclei and the contamination by ER membranes (Matzke *et al.*, 2010). Nonetheless, several Ca^{2+} channels and transporters have been found at the inner and outer membrane of the nuclear envelope, and it is hypothesized that the nuclear pores can act as Ca^{2+}-selective channels. Two interesting examples of nuclear ion channels are Castor and Polux. Initially they had been reported to localize to chloroplasts (Imaizumi-Anraku *et al.*, 2005), but more recent work showed that they are cation channels in the nuclear envelope (Charpentier *et al.*, 2008). Mutation of Castor and Polux abolishes perinuclear Ca^{2+} spiking and, concurrently, root symbioses with arbuscular mycorrhizal fungi and rhizobial bacteria in legume species, such as *Lotus japonicas*, or non-legume species, such as rice (Chen *et al.*, 2009). Root symbiosis in legumes is probably the best example to demonstrate the importance of nuclear calcium signalling and has been extensively reviewed elsewhere (Oldroyd and Downie, 2006; Murray, 2011).

Bacterial calcium signalling

The observation that Ca^{2+} signalling is present in organelles of endosymbiotic origin leads to the question of whether some basic mechanisms of generation or decoding of Ca^{2+} signals can also be found in bacteria. Ca^{2+} signalling is generally considered to be a typical

eukaryotic invention. However, bacteria also need to exclude Ca^{2+} actively from the cytosol in order to operate their phosphate-based metabolism. Similar to eukaryotes, the resulting unequal distribution of free Ca^{2+} between the cytosol and the extracellular space would provide the basis to use Ca^{2+} as a signalling molecule. Furthermore, the discovery of various Ca^{2+} transporters, Ca^{2+}-binding proteins, and Ca^{2+}-dependent processes all argue in favour of some calcium signalling taking place in bacteria.

It has been shown that the intracellular free Ca^{2+} concentration in bacteria is actively maintained in the range of 100-300 nM (Knight *et al.*, 1991; Herbaud *et al.*, 1998;Jones *et al.*, 1999; Torrecilla *et al.*, 2000). Like in eukaryotes, distinct transient changes in the cytoplasmic Ca^{2+} concentration were also observed in bacteria (Dominguez, 2004) for different stimuli such as heat, cold (Torrecilla *et al.*, 2000), salt, osmotic stress (Torrecilla *et al.*, 2001), and oxidative stress (Herbaud *et al.*, 1998), or in response to nitrogen deprivation (Torrecilla *et al.*, 2004; Zhao *et al.*, 2005; Shi *et al.*, 2006; Leganes *et al.*, 2009).

An early bioinformatic investigation revealed the presence of Na^{+}, K^{+}, and Ca^{2+} selective voltage-gated ion channels, Ca^{2+} cation antiporters, and P-type Ca^{2+}-ATPases in 18 bacterial genomes based on sequence homology to eukaryotic counterparts (Paulsen *et al.*, 2000). Since then, experimental evidence for Ca^{2+} transporters in bacteria has accumulated (Raeymaekers *et al.*, 2002;Fujisawa *et al.*, 2009; Faxen *et al.*, 2011). In the cyanobacteria, *Synechococcus elongatus* PCC 7942, the *PacL* gene product was identified as a P-type Ca^{2+}-ATPase (Berkelman *et al.*, 1994), and in *Synechocystis* sp. PCC 6803 the mechanosensitive ion channel MscL was reported to be responsible for major Ca^{2+} effluxes observed after membrane depolarization and high temperature (Nazarenko *et al.*, 2003). Finally, Ca^{2+}/H^{+} antiporters, homologous to the CAX gene family in *Arabidopsis*, have also been identified in cyanobacterial genomes (Waditee *et al.*, 2004). The CAX protein from *Synechocystis* sp. PCC 6803 was shown to be biochemically functional, localized at the plasma membrane, and required for salt tolerance, as well as for adaptation to an alkaline milieu in this strain. In contrast, molecular identities of Ca^{2+}-specific influx systems in bacteria are largely missing so far.

If intracellular Ca^{2+}-mediated signalling should take place in bacteria like in eukaryotic cells, then calcium sensors in the form of Ca^{2+}-binding proteins must exist. During the last decade various successful attempts have been made to identify EF-hand-containing proteins in bacterial genomes (Michiels *et al.*, 2002; Zhou *et al.*, 2006;Rigden *et al.*, 2011). Globally, it can be said that EF-hand-containing proteins are found in most bacterial genomes and that they are evenly distributed throughout families and genera. In addition, new calcium-binding motifs are predicted and found in bacterial proteins, such as the Dx[DN]xDG motif, which is part of the EF-hand motif. This further increases the number of potential Ca^{2+}-binding proteins in bacteria (Morgan *et al.*, 2006; Hu *et al.*, 2011;Rigden *et al.*, 2011). For example, the calmodulin-related protein CasA from the symbiotic bacterium *Rhizobium etli* is secreted during infection and colonization of its plant host (Xi *et al.*, 2000).

How calcium signalling can impact on bacterial physiology is best illustrated by the effect of a Ca^{2+}-binding protein on nitrogen fixation. CcbP, a non-EF hand-containing protein from *Nostoc* (*Anabaena*) sp. PCC7120, has been demonstrated to be an intracellular Ca^{2+}-binding protein which is involved in heterocyst differentiation upon limitation of combined nitrogen (Zhao *et al.*, 2005; Hu *et al.*, 2011). Several genera of cyanobacteria grow heterocysts, which are specialized cells capable of fixing N_2, and they make up a considerable part of the global nitrogen cycle. At the initial stage of differentiation, CcbP becomes degraded by hetR, a serine protease specifically expressed at that stage of development, resulting in a rise of intracellular free [Ca^{2+}] and subsequently heterocyst formation (Shi *et al.*, 2006).

Furthermore, alterations of the nitrogen depletion-induced Ca^{2+} transient led to an arrest of heterocyst differentiation (Torrecilla *et al.*, 2004).

Except for the Ca^{2+} transporters, none of the already described bacterial Ca^{2+}-binding proteins in the literature has clear homologues in higher plant species. The apparent lack of conservation of calcium signalling components is most probably due to the rather different physiology of bacteria and higher plants, whereas the active extrusion of Ca^{2+} by transporters from the cytosol seems to be conserved and universal. Moreover, the eukaryotic cell acquired more complexity in terms of compartmentalization than a bacterial cell, and consequently needs a signal transduction system, such as calcium signalling, to orchestrate all processes in those different subcellular compartments. Interestingly, highly conserved bacterial processes governed by proteins such as MinD and the RelA/SpoT homologue CRSH (Givens *et al.*, 2004; Aldridge and Moller, 2005) might have acquired Ca^{2+} regulation to be able to function coordinately in the cellular environment of higher plants. Table 1 lists 19 conserved proteins between *Arabidopsis thaliana* and *Nostoc* (*Anabaena*) sp. PCC7120, containing the Ca^{2+}-binding Dx[DN]xDG motif. It is interesting that most of these proteins localize, or are predicted to localize to different compartments other than to the plastid. Nevertheless, together with two other proteins, CML41 is predicted to localize to the plastid, which may suggest that the so far ‘missing’ plastidal CaM might be a CaM-like protein.

Conclusions and future perspectives

A central question in Ca^{2+} signalling is how such a simple ion can mount an appropriate and specific physiological response, when almost every stress or developmental process elicits calcium fluxes in the cell. While the answer to the specificity question is manifold (Sanders *et al.*, 2002), it can partly be explained by the compartmentalized nature of calcium signalling. In other words, the possibility to mobilize Ca^{2+} from various subcellular stores, including chloroplasts and mitochondria, combined with its rapid release and uptake, implies that the effect of a Ca^{2+} flux can be highly localized to microdomains in the cytosol (Laude and Simpson, 2009). Furthermore, through the physical separation of organelles from the cytosol, Ca^{2+}-dependent regulation inside organelles, such as chloroplasts, mitochondria, peroxisomes, and nuclei, can be exerted in a highly localized and specific manner.

The question arises then: what is the physiological relevance of the organelles to cellular Ca^{2+} signalling? At least in chloroplasts, the impact of impaired organellar Ca^{2+} handling on plant physiology has already been demonstrated by CAS and PPF1. The mutation of, respectively, a putative chloroplastic Ca^{2+} sensor and a transporter led to impaired stomatal movement and impaired plant growth. To provide further insight into the physiological relevance of organellar Ca^{2+} signalling, it will be crucial to identify the proteins that are responsible for calcium transport (channels and transporters), storage, and decoding of the Ca^{2+} signals that have been observed. Likewise, more should be known on the stimuli that provoke Ca^{2+} signals in mitochondria and peroxisomes, and especially in chloroplasts, where until now only light and dark were found to induce Ca^{2+} fluxes. For future research, the combination of the molecular players and the elicitors of calcium signalling in organelles together with newly generated detection systems for measuring organellar Ca^{2+} concentrations *in planta* (Krebs *et al.*, 2012; Mehlmer *et al.*, 2012) should provide fruitful grounds for further discoveries.

Interplay between organelles can greatly affect Ca^{2+} signalling. In human and animal cells, the intimacy of ER and mitochondria was found to be beneficial for movement of Ca^{2+} between the two stores, and this was further strengthened by the identification of the molecular bridge between them (Kornmann *et al.*, 2009; de Brito and Scorrano, 2010).

Similarly in plants, Ca^{2+} might be exchanged between chloroplasts, mitochondria, and peroxisomes, while they are required to be closely connected for photorespiration and the exchange of metabolites (Majeran *et al.*, 2010; Gowik *et al.*, 2011). Moreover, Ca^{2+} exchange between chloroplasts and the ER cannot be excluded. The co-localization of the ER with stromules—stroma-filled tubules that extend from plastids (Fig. 1)—considerably increases the interactive surface between the plastid and the ER (Schattat *et al.*, 2011*a*, b). The observation that sugars and abiotic stresses induce stromule formation (Gray *et al.*, 2012; Schattat and Klosgen, 2011) further supports the idea that Ca^{2+} signals could be modulated by the interplay between organelles.

The data on bacterial calcium signalling clearly indicate that the prerequisites for eukaryotic Ca^{2+} signalling, an actively maintained Ca^{2+} gradient across membranes and Ca^{2+} sensor proteins, are already present in bacteria. The even distribution and frequent occurrence of Ca^{2+} transporters and EF-hand-containing proteins throughout genomes of bacterial families (Paulsen *et al.*, 2000; Zhou *et al.*, 2006) further indicate a prokaryotic origin of these core elements in eukaryotic Ca^{2+} signalling. For this, it is reasonable to search for conserved Ca^{2+} signalling processes between bacteria, eukaryotes, and their endosymbionts, mitochondria and plastids, too.

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Abbreviations

ABA	abscisic acid
CAS	calcium sensing
CDPK	calcium-dependent protein kinase
PM	plasma membrane

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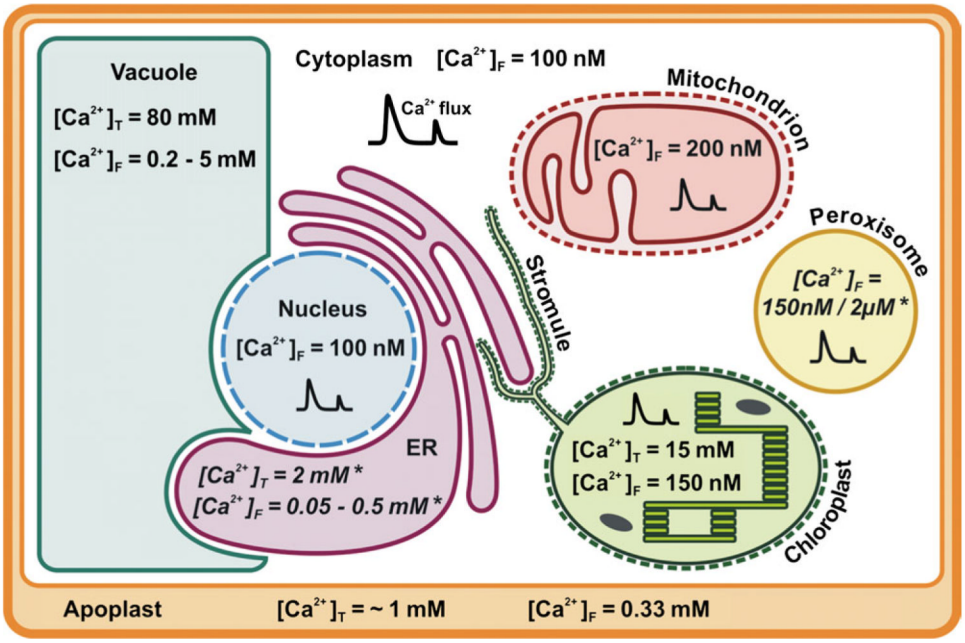

Fig. 1. Summary of organellar Ca^{2+} concentrations in the plant cell.

Values for reported total ($[Ca^{2+}]_T$) and free resting ($[Ca^{2+}]_F$) Ca^{2+} concentrations in the different organelles (apoplast, cytoplasm, vacuole, nucleus, ER, chloroplast, mitochondrion, and peroxisome) that were mentioned in the text are depicted here. The values are approximate values and probably vary depending on the tissue or plant species, but nevertheless they provide a general impression of Ca^{2+} levels across the cell. For ER and peroxisomes, no data on Ca^{2+} concentration in plants are available. The given values are taken from the animal field and marked with an (*). Calcium fluxes are illustrated by a double peak-shaped symbol (ᔑ).

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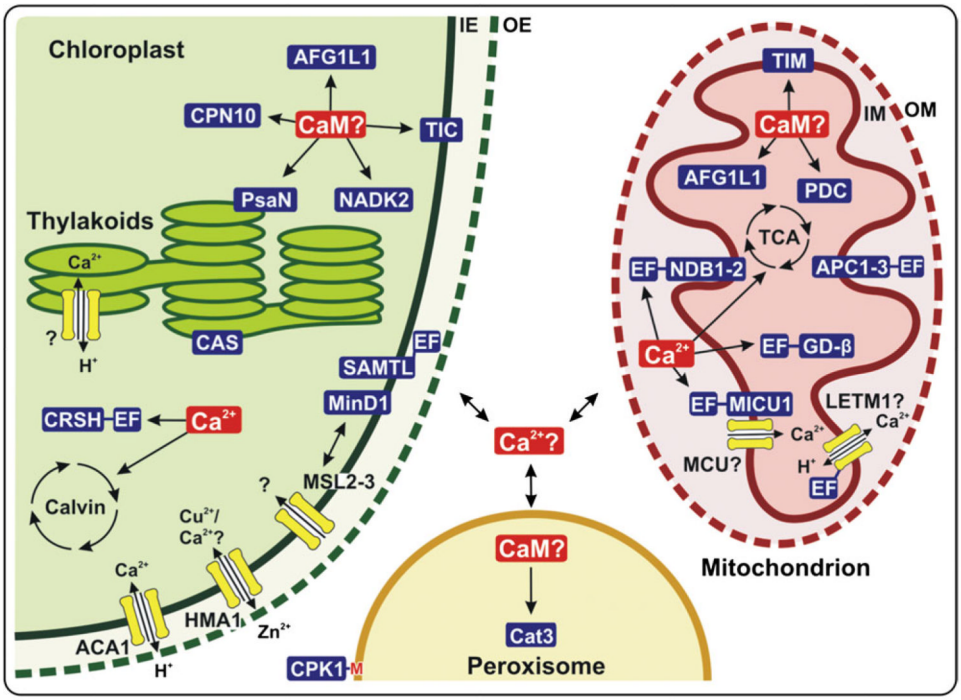

Fig. 2. Overview of proteins involved in calcium signalling in chloroplasts, mitochondria, and peroxisomes of plants.

Calcium and calmodulin (CaM) are highlighted by red boxes. Calcium transporters are depicted in yellow, and calcium/CaM-binding proteins in blue. EF-hand-containing proteins are indicated by the presence of an 'EF'-box. Proteins of uncertain nature (lack of protein identity or uncertain function) are indicated with a (?). The central 'Ca^{2+}?' symbol indicates the possibility of calcium exchange between organelles and the contribution of organelles to the cytoplasmic calcium concentration. Full names for protein abbreviations can be found in the text. Other abbreviations are Calvin (Calvin–Benson cycle), TCA (tricarboxylic acid cycle), IE (inner envelope), OE (outer envelope), IM (inner membrane), OM (outer membrane).

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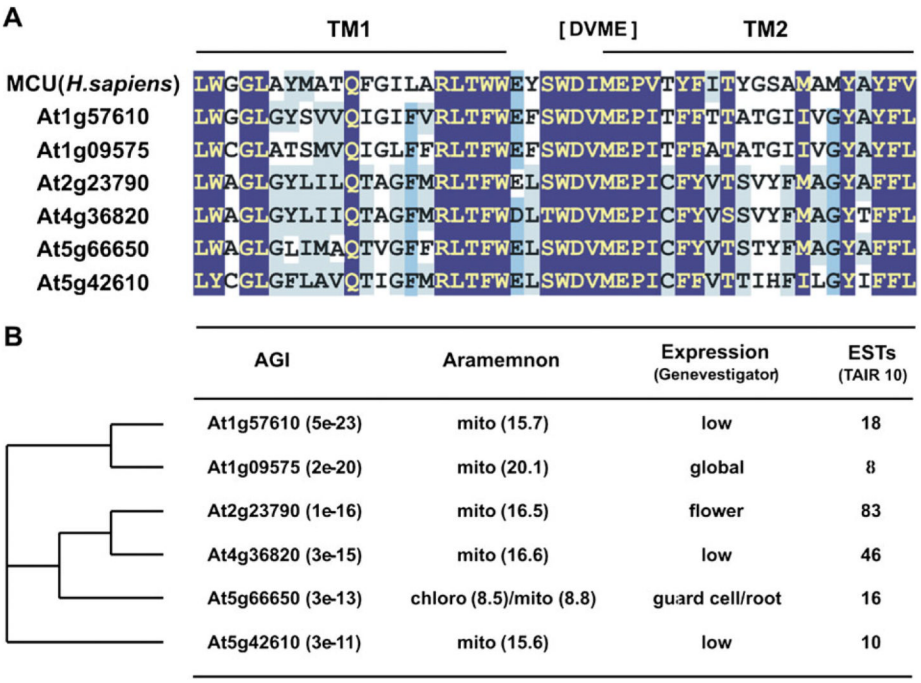

Fig. 3. Conserved regulators of mitochondrial Ca^{2+} import between humans and plants.
(A) Alignment of the transmembrane domain of the human mitochondrial calcium uniporter (MCU, *gi24308400*) with its six homologues from *Arabidopsis thaliana* displays the two transmembrane helices (TM1 and TM2) connected by a highly conserved loop domain containing the amino acids DIME in human and DVME in *Arabidopsis*. (B) Overview of targeting and expression of the MCU homologues. The cladogram displays the homology relationship between the *Arabidopsis* proteins. The blast e-value for each protein relative to the human MCU is written in parentheses after the *Arabidopsis* gene identifier (AGI). Protein localization was predicted by Aramemnon (Schwacke et al., 2003) and the meta-score is indicated in parentheses. Organ-related RNA expression profiles were examined with the help of the GENEVESTIGATOR browser (Zimmermann et al., 2004) and the presence of expressed sequence tags (ESTs) in the TAIR 10 database (www.arabidopsis.org).

Table 1

Arabidopsis proteins of putative cyanobacterial origin containing the Ca^{2+} binding Dx[DN]xDG motif

AGI code	Annotation	Aramemnon prediction[a]			MS/MS[b]
		Plastids	Mitochondria	Secretory pathway	
At3g12290	Amino acid dehydrogenase family protein	1.2	0	0	PM, plastid
At3g09090	DEX1 defective in exine formation	0	3.3	28.2	PM
At2g34020	Calcium-binding EF-hand family protein	5	6.2	16	
At3g50770	CML41 calmodulin-like 41	11.4	2.3	0	
At4g10060	β-Glucosidase GBA2 type family protein	0	0	0	
At1g33700	β-Glucosidase GBA2 type family protein	0	0	0	PM
At5g49900	β-Glucosidase GBA2 type family protein	0	0	0.8	Vacuole
At1g74960	KAS2 fatty acid biosynthesis 1	27.2	0	7.5	Plastid
At2g04540	mtKAS1 β-ketoacyl synthase	2.2	19	0.5	
At1g62810	Copper amine oxidase family protein	0.4	0	30.6	
At1g31690	Copper amine oxidase family protein	3.3	0	27.7	Peroxisomes
At3g43670	Copper amine oxidase family protein	0.1	0	35.2	
At1g31670	Copper amine oxidase family protein	0	0	29.1	
At4g12290	Copper amine oxidase family protein	2.2	0	24.3	PM
At4g14940	AO1 amine oxidase 1	0	0	28.9	Extracellular
At3g48560	CSR1 Chlorsulphuron/imidazolinone resistant 1	30.6	0.6	0	Plastid
At5g04130	GYRB2 DNA gyrase B2	3.6	18.9	2.6	Mitochondrion
At3g10270	GYRB1 DNA gyrase B1	0	0	0	Mitochondrion
At3g23890	TOPII topoisomerase II	4	3.2	0	

PM, plasma membrane.

[a] Aramemnon score refers to the likelihood of localization for a protein predicted with Aramemnon (Schwacke et al., 2003).

[b] MS/MS indicates evidence for the localization of the protein in a cellular compartment based on proteomics studies summarized in the SUBA database (Heazlewood et al., 2007).