Calcium: Not Just Another Ion

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Abstract Calcium (Ca^{2+}) represents very likely the most versatile ion in living organisms. It is involved in nearly all aspects of plant development and participates in a plethora of regulatory processes. Calcium is an important signaling compound, regulates cellular metabolism and is important for endocytosis and exocytosis. Calcium can easily form complexes with proteins, membranes and other organic acids rendering this ion a versatile signaling constituent and simultaneously a toxic cellular compound. Consequently, the required tight spatial and temporal control of intracellular Ca^{2+} levels provided the basis for the emergence of calcium signaling. It is this apparent antagonism between the obvious cellular abundance of Ca^{2+} as a structural important ion in the plant and its required rareness in the cytoplasm as well as the evident question how this simple ion can specifically function in such a myriad of distinct process that has sparked considerable interest and research. Here we will discuss new insights into the signaling function of Ca^{2+} in the context of its diverse cell biological roles.

1 Introduction

Calcium (Ca^{2+}) represents very likely the most versatile ion in living organisms. It is involved in nearly all aspects of plant development and participates in a plethora of regulatory processes. Calcium is an important signaling compound, regulates cellular metabolism and is important for endocytosis and exocytosis. Moreover, it is important for energy production within mitochondria and chloroplasts. Ca^{2+} is an ion that due to its specific properties can easily be dehydrated. Because of its

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flexibility in forming different coordination numbers and complex geometries, Ca^{2+} can easily form complexes with proteins, membranes and other organic acids like citrate and oxalate. This unique feature renders Ca^{2+} a toxic cellular compound because it can easily form insoluble complexes with phosphate (and consequently ATP), and also with DNA and RNA. However, on the other hand this property enabled the evolution of Ca^{2+} as an important signaling molecule because the required tight spatial and temporal control of intracellular Ca^{2+} levels provided the basis for the emergence of calcium signaling. It is this apparent antagonism between the obvious cellular abundance of Ca^{2+} as a structural important ion in certain organelles and cell structures and its required rareness in the cytoplasm as well as the yet not solved question how this simple ion can specifically function in such a myriad of distinct process that has sparked considerable interest and research. Several excellent reviews have in the past addressed the nutritional and signaling functions of Ca^{2+} (Hepler and Wayne 1985; Sanders et al. 2002; White and Broadley 2003; Hetherington and Brownlee 2004; Hirschi 2004). Here we will discuss new insights into the signaling function of Ca^{2+} in the context of its diverse cell biological roles.

2 Nutritional and Structural Functions of Ca^{2+}

2.1 Nutritional Functions of Ca^{2+}

Work in the late forties of the last century established that Ca^{2+} constitutes an important macronutrient for plant germination, growth and development (Mulder 1950; Helms 1971). Besides Mg^{2+}, Ca^{2+} is the most abundant group two element in plants and in different plant species Ca^{2+} can reach concentrations between 0.02% (e.g., Poacea species) and ~5% of the plant dry weight, with higher concentrations generally found in dicotylous plants (Broadley et al. 2003). In general, plants are classified into calcifuges that grow on soil with low Ca^{2+} content, and calcicoles, growing on high calcareous soils that can tolerate a high soluble Ca^{2+} concentration like Brassicaceae (White and Broadley 2003). Some calcicoles plants are able to deposit large amounts of Ca^{2+} in trichomes to protect the stomata from excessive Ca^{2+} (De Silva et al. 1996). Within the plant, Ca^{2+} is unequally distributed, and reaches higher concentrations in leaves and stems than in underground tissues (Demarty et al. 1984). In aerial parts, young leaves and fruits contain lower Ca^{2+} concentrations than older leaves (Kirkby and Pilbeam 1984). Calcium uptake is confined to the root tip or to nodes of newly emerging lateral roots, indicating that Ca^{2+} does not cross the "casparian strip" and follows the apoplastic pathway to reach the xylem of the central cylinder (Kirkby and Pilbeam 1984).

Calcium deficiency affects every tissue and plant organ. Generally, it causes stunted plant growth and appearance of brown spots associated with polyphenol

oxidation, necrosis of primarily meristematic cells and later on in older tissues, cracking of fruits and curling of the leaves (Bussler 1962; Simon 1978). Characteristic symptoms are "tipburn" mostly described in agricultural vegetables like lettuce, or "blossom-end rot" in fruits of tomato (Simon 1978; Ho and White 2005). Especially fast growing young tissues with a low transpiration rate and therefore insufficient supply of Ca^{2+} from the xylem are prone to deficiencies and exhibit symptoms early. This effect is further enhanced by the relative immobility of Ca^{2+} within the plant because Ca^{2+} is insufficiently redistributed from older leaves, which harbor a higher Ca^{2+} content (van Goor and Wiersma 1974; White and Broadley 2003).

Root growth and the development of the root system is also affected under Ca^{2+} deficiency leading to growth arrest and finally stalling of the root (Simon 1978). Ca^{2+} also affects the uptake of other ions into the root system. It improves the selective uptake of K^+ in the presence of high Na^+ concentrations (Epstein 1961,1998). Here Ca^{2+} concurrently blocks Na^+-permeable channels and reduces Na^+ uptake (Demidchik and Tester 2002) and inhibits Na^+ induced K^+ efflux (Shabala et al. 2006). In addition, it was suggested that Ca^{2+} reduces Na^+ induced depolarization of the plasma membrane thereby minimizing K^+ efflux by depolarization-activated channels (Shabala et al. 2006).

2.2 *Structural Functions of Ca^{2+}*

The large ion radius of Ca^{2+} facilitates straightforward dehydration of the ion thereby enabling the binding of Ca^{2+} to several anionic headgroups of membrane-lipids like phosphatidylserine, phosphatidic acid or glycolipids and additionally to membrane proteins (Hauser et al. 1976; Jaiswal 2001). Consequently, Ca^{2+} represents an important regulator of different dynamic membrane processes. Within the cell, Ca^{2+} promotes the fusion of vesicles to their target membranes by cross-linking the lipids from the two different membranes (Williams 1970; Hauser et al. 1976). On the other hand, Ca^{2+} is a structure forming cation. It reduces the fluidity and therefore enables a tighter packaging of the membrane lipid bilayer, thereby reducing passive ion-fluxes of monovalent cations like H^+, Na^+ and K^+ (Williams 1970; Jaiswal 2001; White and Broadley 2003; Plieth 2005). Therefore, Ca^{2+} deficiency induces membrane leakyness for monovalent cations rendering plants susceptible to damage by salt or low pH (Plieth 2005).

Calcium is also an abundant cell wall component critically regulating the strength and pH of the cell wall (Demarty et al. 1984). Here, Ca^{2+} bridges cell wall polymers like negatively charged galacturonanes or xylans (Brett and Waldron 1996) and influences the formation of the 1,3-β-glucan "callose", which is an essential cell wall component in the cell plate of dividing cells, in growing pollen tubes, or is produced after wounding. Remarkably, the enzyme β-1,3 glucan synthase, which forms callose is directly activated by Ca^{2+} (Kauss 1987; Brett and Waldron 1996).

3 The Evolution of Ca^{2+} as a Signaling Molecule

Several physico-chemical features are unique to Ca^{2+} and predestinate this ion as potential signaling molecule. Due to the large ionic radius of a Ca^{2+} ion (99 pm) water molecules are less tightly bound to Ca^{2+} than to the smaller Mg^{2+} ion (65 pm). Therefore, less energy is required to remove the bound water to enable Ca^{2+} ligand interaction (Hepler and Wayne 1985). Consequently, Ca^{2+} ions are easily dehydrated, and can form complexes with high and variable coordination numbers (6–8, but also 5–10) and are flexible in coordination geometry and Ca^{2+}-ligand distances. Ca^{2+} binds favorably to carboxylate oxygen, while Mg^{2+} or Zn^{2+} have higher affinity toward nitrogen-based ligands, associated with amino acids, which are not as common in proteins than glutamate and aspartate (Williams 1970; Ochiai 1991). Although other ions like Ba^{2+}, Sr^{2+} and Zn^{2+} could substitute for Ca^{2+} function, they are less abundant than Ca^{2+} and other divalent cations like Cd^{2+} and Pb^{2+} are highly toxic (Jaiswal 2001).

On the other hand, these features of Ca^{2+} are also the reason why elevated levels of Ca^{2+} will react with inorganic phosphate, forming an insoluble precipitate. Therefore, high Ca^{2+} acts as a cytotoxin inhibiting the phosphate-based energy system (Hepler and Wayne 1985). Moreover, excess Ca^{2+} would compete with Mg^{2+} for binding sites on various proteins. Thus, during evolution, organisms were forced to evolve de-toxifying mechanisms that are effective in keeping Ca^{2+} at low levels in the cytoplasm. Importantly, Ca^{2+} pumps, which are responsible to extrude Ca^{2+} out of the cytoplasm, are themselves Ca^{2+}-activated resulting in a time lag of efflux activation that follows the entry of Ca^{2+} into the cytoplasm. This facet of Ca^{2+} regulation mandatory leads to a transient Ca^{2+} elevation within the cell when Ca^{2+} permeable channels open (Ochiai 1991) thereby providing a unique system to evolve the extant Ca^{2+}-regulated circuits in which Ca^{2+} itself activates Ca^{2+} channels within the cell. This Ca^{2+}-dependent release of Ca^{2+} from external or internal stores now is responsible for a rapid, amplified (100 fold) but still transient Ca^{2+} increase and forms the mechanistic basis of many Ca^{2+} signaling events (Williams 2004).

Since the concentration of Ca^{2+} in the cytoplasm is kept at low levels, the concentration can be, rapidly and efficiently modulated. In contrast, due to physiological requirements, the cytosolic concentrations of Mg^{2+} or K^{+} are relatively high. Therefore, a 100 fold change of these ions in the cytosol would be more difficult to achieve: firstly the amount of ions to be transported would be much higher and secondly, the resulting dramatic change in cellular ion homeostasis would be detrimental while a 100 fold change of the Ca^{2+} concentration only marginally effects the osmotic balance of the cytoplasm (Hepler and Wayne 1985; Ochiai 1991). Finally, due to the unique properties of Ca^{2+}, this ion can bind to and dissociate from proteins much faster than other ions (Ochiai 1991). This aspect of Ca^{2+}, allows for transient interaction with calcium binding proteins and has enabled the evolution of a signaling system that can control fast and diverse reactions (Hepler and Wayne 1985).

4 Calcium Release in Response to Signals and Stimuli

4.1 Calcium Responses to Abiotic, Biotic Factors and Development

Cytoplasmic changes of Ca^{2+} concentration from resting concentrations of 100–300 nM up to 1 μM are observed after various signals or stimuli. Abiotic cues like salt (Lynch et al. 1989), osmotic stress (Takahashi et al. 1997; Cessna et al. 1998), drought (Knight et al. 1998), ozone (Clayton et al. 1999), anoxia (Subbaiah et al. 1994), CO_2 (Webb et al. 1996), gravitation (Lee et al. 1983; Gehring et al. 1990; Fasano et al. 2002), mechanical injury and touch (Haley et al. 1995; Legue et al. 1997) all cause transient elevations in Ca^{2+} concentration. Different temperature regimes can also induce Ca^{2+} responses. Cold stress, especially the cooling rate, is reflected by specific increases of intracellular Ca^{2+} (Knight et al. 1991; Plieth et al. 1999). Development of freezing tolerance requires influx of extracellular Ca^{2+} and enhanced Ca^{2+}-dependent exocytosis to enable resealing of the membrane after mechanical disruption provoked by cold (Schapire et al. 2008; Yamazaki et al. 2008). These membrane fusions are mediated by Ca^{2+} interaction with the sensor protein synaptotagmin to promote interaction with SNARE proteins (Kesavan et al. 2007; Schapire et al. 2008; Yamazaki et al. 2008). Furthermore, tobacco plants also respond with Ca^{2+} release to heat shock (Gong et al. 1998), while Ca^{2+} transients were recorded in Arabidopsis during the recovery from heat exposure (Larkindale and Knight 2002).

Also light responses in plants are accompanied by complex and specific patterns of Ca^{2+} transients. Changes in cytoplasmic Ca^{2+} concentration were observed after plant exposure to red but not far-red light implicating that this reaction is mediated by the photoreceptor phytochrome B (Shacklock et al. 1992; Neuhaus et al. 1993). Blue light Ca^{2+} responses depend on the phototropin photoreceptors but not on cryptochromes (Baum et al. 1999; Harada et al. 2003; Stoelzle et al. 2003). However, cryptochromes could fine tune a Ca^{2+} response (Long and Jenkins 1998), and cryptochrome signaling is mediated via the Ca^{2+}- binding protein SUB1 (short under blue light), which in turn modulates phyotochrome function (Guo et al. 2001a). These observations exemplify the complexity and interconnection of Ca^{2+} responses that can occur in reaction to a single environmental cue like light. In addition, Ca^{2+} is also released in a circadian manner and responds distinctly to light intensity (Love et al. 2004).

Similar to abiotic stimuli, biotic factors like bacterial pathogens (Atkinson et al. 1990; Xu and Heath 1998; Blume et al. 2000), fungal elicitors (Knight et al. 1991), attacks by herbivores or symbiotic interactions with nitrogen-fixing bacteria or mycorrhizal fungi cause different and specific Ca^{2+} responses (Ehrhardt et al. 1996; Kosuta et al. 2008). Moreover, cell apoptosis during the hypersensitive response induced by a pathogen is mediated by a complex regulation of cellular Ca^{2+} dynamics. An initial transient increase of Ca^{2+} occurs after pathogen infection

(Levine et al. 1996) and is then followed by a silent phase and a second, but sustained increase of cytoplasmic Ca^{2+} (Grant et al. 2000). Finally, a massive efflux of Ca^{2+} to the apoplast leads to cell collapse and death (Nemchinov et al. 2008).

Transient changes in cellular Ca^{2+} concentration have also extensively been observed during tightly regulated developmental processes. Oscillatory, tip-localized gradients of Ca^{2+} are important for proper growth of pollen tubes and expansion of root hairs by enabling high exocytotic turnover at the growing tip (Rathore et al. 1991; Miller et al. 1992; Rudd and Franklin-Tong 1999). In contrast, the self-incompatibility response during pollination is mediated by a rise of Ca^{2+} originating from the nuclear region, potentially regulating gene expression to inhibit pollen tube growth (Franklin-Tong et al. 1993). Ca^{2+} is also essential for fertilization (Faure et al. 1994), and a Ca^{2+} transient lasting for several minutes has been observed during the sperm-egg fusion event (Digonnet et al. 1997).

4.2 Calcium Responses to Hormones

Calcium release and subsequent signal transduction events are important after perception of phytohormones like cytokinin (Hahm and Saunders 1991), salicylic acid (Kawano et al. 1998) and ethylene (Raz and Fluhr 1992). Jasmonic acid (Sun et al. 2006) and its precursor 12-oxophytodienoic acid (OPDA) induce a large increase of cytoplasmic Ca^{2+} (Walter et al. 2007). Giberellic acid (GA) causes a long sustained increase of cytosolic Ca^{2+} at the cell periphery by influx of extracellular Ca^{2+} (Gilroy and Jones 1992), but the release of intracellular Ca^{2+} is also important for induction of α-amylase transcription (Chen et al. 1997). GA mediated calcium influx also precedes and is important for the secretion of α-amylase (Bush 1996) and can be accounted to the effect that Ca^{2+} enhances exocytosis (Homann and Tester 1997) by promoting membrane fusion (Bhalla et al. 2006; Martens et al. 2007). Additionally, GA also increases Ca^{2+} within the endoplasmic reticulum (ER), which is important for the maturation of α-amylase (Bush et al. 1989a, b). On the other hand, GA induced Ca^{2+} release in aleurone cells can be reversed by ABA (Gilroy and Jones 1992), and Ca^{2+} uptake into the ER is also inhibited by ABA (Bush et al. 1993).

Auxin induces an oscillatory Ca^{2+} release and thereby promotes stomatal opening (Felle 1988; Irving et al. 1992). Abscisic acid (ABA) also induces oscillatory Ca^{2+} transients resulting in closure of the stomata and being essential to keep them closed in the long term (McAinsh et al. 1990; Allen et al. 1999, 2000, 2001; Staxen et al. 1999). In another study, spontaneous Ca^{2+} oscillations were terminated by ABA in some guard cells (Klusener et al. 2002). These findings suggest that ABA can have positive and negative effects on the cellular Ca^{2+} level. Moreover, since Ca^{2+} levels in the cytosol exhibit circadian oscillation, these different levels of cytoplasmic calcium could also differentially prime the guard cells and other cell types to the effect of ABA, which then leads to a different final outcome (Dodd et al. 2005). Indeed, MacRobbie (1989) found out that the stimulatory effect of ABA is

stronger in the afternoon than in the morning (MacRobbie 1989). Moreover, the parameters of Ca^{2+} oscillations which lead to stomatal closure could depend on the physiological condition of the plant (Klusener et al. 2002).

These examples clearly illustrate the universality and complexity of Ca^{2+} responses as well as their intricate and often confusing interconnection with various cellular or hormonal processes. They also highlight a continuing dilemma of plant Ca^{2+} research in which a further accumulation of descriptive data linking Ca^{2+} responses to certain biological processes will not likely advance our understanding of the underlying functional principles and causalities.

4.3 Interconnection of Ca^{2+} Dynamics with other Second Messengers

A similar complex situation applies to the interconnection of Ca^{2+} with other second messenger components. Various second messengers induce Ca^{2+} release, and their generation itself is often regulated by Ca^{2+}. Diacylglycerol (DAG), Cyclic nucleotides like cAMP and cGMP (Volotovski et al. 1998), cyclic Adenosine-diphospho-Ribose (cADPR) (Allen et al. 1995), Inositol-3-phosphate (InsP3) (Alexandre 1990; Gilroy et al. 1990) or its derivate myo-Inositol-Hexakisphosphate (InsP6) (Lemtiri-Chlieh et al. 2003), Nicotinic Acid-Adenine Dinucleotidphosphate (NAADP) (Navazio et al. 2000), Sphingosine-1-phosphate (S1P) (Ng et al. 2001), extracellular Glutamate (Dennison and Spalding 2000) and extracellular ATP (Demidchik et al. 2003, 2009; Jeter et al. 2004) can mobilize Ca^{2+} either from intracellular or extracellular stores, respectively. Reactive oxygen species (ROS), generated by plasma membrane NADPH oxidases, cause a Ca^{2+} influx into the cytosol (Price et al. 1994; Pei et al. 2000; Foreman et al. 2003). Moreover, different types of ROS can differentially activate Ca^{2+} permeable channels in different root tissues (Demidchik et al. 2007). Like ROS, the Ca^{2+} releasing second messenger cADPR is also produced after application of ABA (Wu et al. 1997; Leckie et al. 1998), and is also synthesized in a circadian manner which could be important for the observed circadian Ca^{2+} oscillations (Dodd et al. 2007). These observations point to a further level of complexity interconnecting Ca^{2+} simultaneously with second messengers, hormonal responses and reactions to environmental cues.

5 Organelles and Ca^{2+}

Calcium can rapidly enter the cell (10^6 molecules/sec. per channel) but diffusion within the cytoplasm is very limited (up to 0.5 μm) and Ca^{2+} is rapidly bound by Ca^{2+} binding proteins or buffered by cell organelles (within 50 μsec) (Clapham 1995; Lecourieux et al. 2002). Indeed, near membrane Ca^{2+} concentrations in the

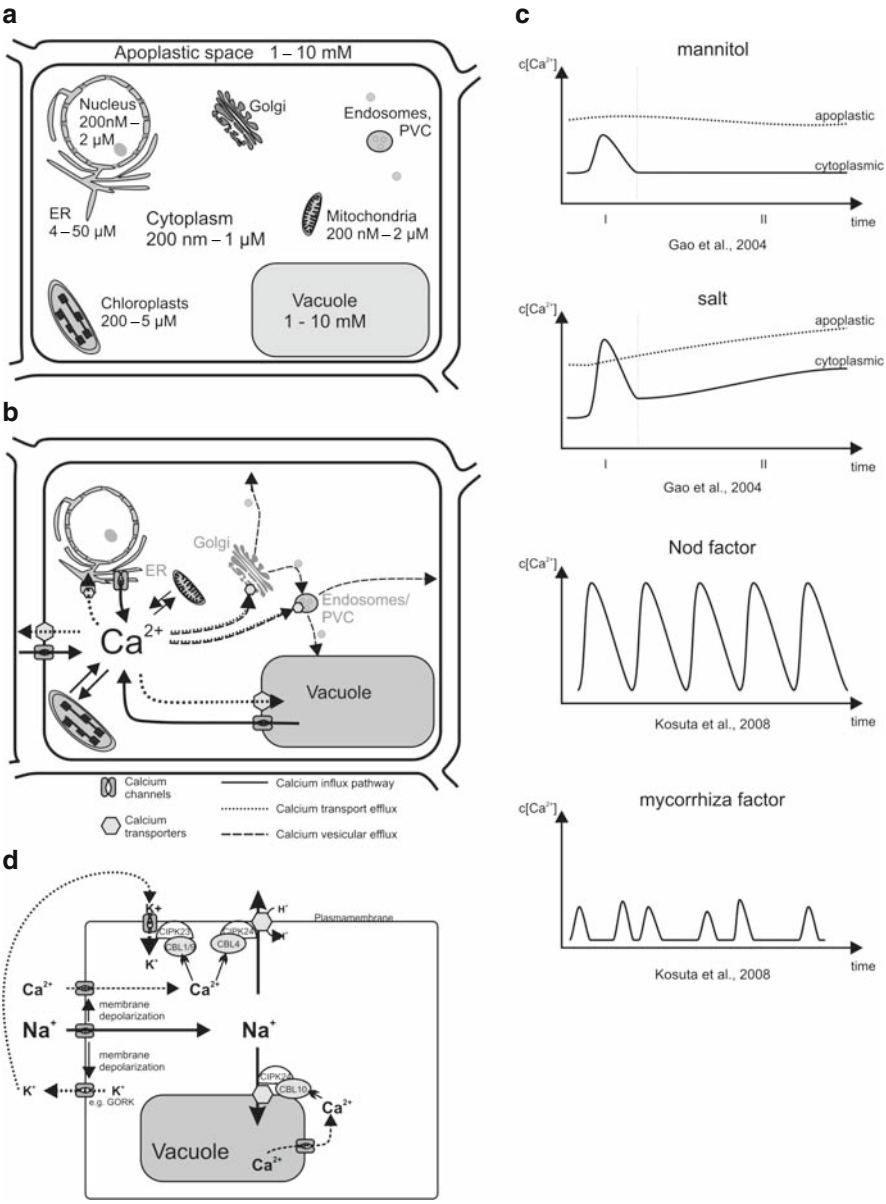

Fig. 1 (**a**) Distribution and concentration of free Ca^{2+} generally found in different compartments of the cell. Highest concentrations of calcium are found in the apoplastic space and within the vacuole (up to 10 mM). Also the ER can take up large amounts of Ca^{2+} (up to 50 µM). Within mitochondria and chloroplasts, Ca^{2+} concentrations can reach up to 2 and 5 µM. In the nucleoplasma and cytoplasma, the resting Ca^{2+} concentrations are maintained around 200 nM. The concentrations of Ca^{2+} within the Golgi and Endosomes are not known. (**b**) Different cellular compartments contribute to the cytoplasmic rise of Calcium. Influx is mainly regulated via influx

vicinity of Ca^{2+} channels are estimated to be 10–100 fold higher than the measured cytoplasmic Ca^{2+} concentrations (Etter et al. 1996; Demuro and Parker 2006). Therefore, local Ca^{2+} signals at specific microdomains are assumed to be the basis of differential Ca^{2+} signals, which can promote different responses to various signals (Berridge 2006).

Various organelles and compartments are implicated in different Ca^{2+} responses, and moreover, several compartments can act in concert to shape a Ca^{2+} signal and to establish a correct response to the signal. The major Ca^{2+} store of the cell is the apoplast which supplies extracellular Ca^{2+} (1–10 mM). From the apoplast, Ca^{2+} is released by Ca^{2+} channels which are activated by changes of the plasma membrane voltage, or by ligand activated channels (Fig. 1a).

Intracellular Ca^{2+} is stored mainly in the vacuole and ER, and released by voltage dependent and ligand gated channels. Within the vacuole, Ca^{2+} functions as counterion of inorganic and organic anions (White and Broadley 2003). Additionally, different types of vesicles, like Golgi or endosomal vesicles are potential mobile calcium stores (Wagner and Rossbacher 1980; Wick and Hepler 1980; Sakai-Wada and Yagi 1993; Trewavas 1999; Li et al. 2008), or transport excess calcium and other cations out of the cytoplasm either to the apoplast or to the vacuole (Fig. 1b) (Menteyne et al. 2006). Other organelles, like chloroplast and mitochondria, store Ca^{2+} also and are important to maintain the cellular Ca^{2+} and ATP homeostasis (Plieth 2005).

5.1 Calcium Signaling within the Nucleus

High levels of calcium are observed in the nucleus and the nuclear envelope (Wick and Hepler 1980). The nucleus can respond specifically and independently of the cytoplasm to a signal by specific temporal changes in Ca^{2+} concentration

Fig. 1 (continued) from the apoplastic space or vacuole. Also the ER, and organelles like chloroplasts and mitochondria contribute to the influx of cytosolic Ca^{2+}. The contribution of Golgi and Endosomes to Ca^{2+} influx is currently not known, but they can contribute to the uptake of Ca^{2+} out of the cytoplasma. In addition, Golgi and Endosomes can mediate the export of Ca^{2+} to the apoplast or vacuole. (**c**) Different stress stimuli provoke specific calcium signatures. Application of mannitol or sodium provokes a transient initial (I) rise of cytoplasmic Ca^{2+} concentrations, which is lower in the case of mannitol compared sodium induced responses. A second phase (II) of sustained Ca^{2+} increase is observed after sodium application, but not after mannitol treatment. Moreover, apoplastic Ca^{2+} concentrations rise also under sodium stress, but not in response to mannitol stress. Additionally, different symbiotic factors induce distinct Ca^{2+} spiking pattern. While Nod factors mediate a repetitive, constant Ca^{2+} spiking, mycorrhiza provokes a non-uniform, chaotic Ca^{2+} spiking, which exhibits lower maximal amplitudes. (**d**) Model of CBL/CIPK function in salt stress responses. During Na^+ stress, sodium mediates influx of Ca^{2+} from the apoplastic space. While apoplastic Ca^{2+} triggers CBL4/CIPK24 to activate the sodium extrusion exchanger SOS1 to directly transport sodium out of the cell, CBL10 together with CIPK24 could be activated by vacuolar Ca^{2+} to transport excess Na^+ into the vacuole. Additionally, K^+ ions that are released due to depolarisation of the plasma membrane by Na^+ could be transported back into the cell by activation of CBL1/CBL9/CIPK23 which in turn activate K^+ inward rectifying channels

(van Der Luit et al. 1999; Pauly et al. 2001). Bacterial elicitors induce a Ca^{2+} release in the cytosol and nucleus. However, the nitric oxide (NO) signal following elicitor application is important for the Ca^{2+} release in the cytosol, but it does not trigger a nuclear Ca^{2+} response (Lamotte et al. 2004). The nucleus also exhibits specific calcium signals in response to different elicitors. Harpin and flagellin resulted in a different Ca^{2+} release than observed in response to carbohydrate elicitors like oligogalacturonides. Remarkably, the cytosolic Ca^{2+} response to these elicitors was comparable (Lecourieux et al. 2005).

These observations suggest that the nucleus does indeed harbor an independent Ca^{2+} machinery which could involve P-ATPases and nucleotide gated channels located at the inner membrane of the nucleus to regulate the nuclear Ca^{2+} reservoir (Mazars et al. 2009). Although it was reported that nuclei are Ca^{2+} impermeable (Xiong et al. 2004), inhibition of Ca^{2+} entry from the extracellular milieu prevented nuclear Ca^{2+} rises (Pauly et al. 2001; Mazars et al. 2009). In additon, it was reported that the nuclear membrane can take up Ca^{2+} in an ATP-dependent manner, which then can be released into the nucleus, implicating that nuclei harbor autonomous Ca^{2+} machineries (Bunney et al. 2000).

In legumes, Nod factor-mediated perinuclear oscillations of Ca^{2+} that occur 10-30 minutes after the initial Ca^{2+} rise in the cytosol (Ehrhardt et al. 1996; Felle et al. 1999), are mediated by the proteins CASTOR and POLLUX, which resemble bacterial potassium channels. However, the exact function of these proteins has remained unclear (Charpentier et al. 2008).

5.2 Calcium Regulation by the ER

Especially due to its large surface within the cell the ER is likely to represent an important intracellular Ca^{2+} store. The ER contains different types of Ca^{2+} channels and transporters, to regulate cytoplasmic and luminal Ca^{2+} levels. However, no ER-localized Ca^{2+} channel from plants has been identified at the molecular level and our understanding of the contribution of the ER to cellular dynamics of Ca^{2+} is much less advanced than in animal systems. Considering the absence of channels that exhibit recognizable similarity to animal InsP3 and Ryanodine-receptors in plants (with the exception of Chlamydomonas and Volvox (Wheeler and Brownlee 2008)) the interconnection of the ER with the cellular Ca^{2+} homeostasis in plants may be fundamentally different than in animals cells.

The ER is loosely associated with the mitotic apparatus, and Ca^{2+} levels regulated by the ER could be therefore important to control cell division (Hepler 2005). Within the lumen of the ER, calcium is important for the maturation of proteins (Bush et al. 1989b). Ca^{2+} concentration within the lumen of the ER is tightly controlled by Calreticulin (CRT), a high capacity calcium binding protein (25 calcium ions/protein) (Persson et al. 2001). Overexpression or reducing the expression either enhances resistance to Ca^{2+} depletion or leads to increased sensitivity to low Ca^{2+}, respectively (Persson et al. 2001). Moreover, increasing the Ca^{2+} buffer

capacity by overexpressing CRT also enhances the stimulus induced Ca^{2+} release from the ER (Persson et al. 2001).

5.3 Mitochondrial Calcium Dynamics

Mitochondria have to retain high concentrations of Ca^{2+} to maintain the activity of enzymes like the NADH dehydrogenase (Moore and Åkerman 1984). Moreover, mitochondria are able to take up enormous amounts of Ca^{2+} from the cytosol (Dieter and Marme 1980). The resting concentration of Ca^{2+} in mitochondria has been estimated to be around 200 nM (Subbaiah et al. 1998; Logan and Knight 2003), and the mitochondrial Ca^{2+} content increases dramatically in response to cytoplasmic Ca^{2+} rises (Logan and Knight 2003). Therefore, mitochondria represent important cellular Ca^{2+} sinks that can contribute to the reduction of cytosolic Ca^{2+} levels after a stimulus induced elevation of cytoplasmic Ca^{2+} concentration (Bygrave 1978; Dieter and Marme 1980, 1983).

In addition, specific stimuli like touch or hydrogen peroxide induce a transient increase of mitochondrial Ca^{2+} concentration that was suggested to occur independently of the cytosolic rise of Ca^{2+}, pointing to a semi-autonomous mitochondrial calcium signaling pathway (Logan and Knight 2003). Moreover, mitochondria can also contribute to the cytosolic rise of Ca^{2+} by release of Ca^{2+} during anoxic conditions (Subbaiah et al. 1998).

5.4 The Role of Chloroplasts in Cellular Calcium Homeostasis

Chloroplasts can accumulate Ca^{2+} in the millimolar range and thereby can contribute to the cellular Ca^{2+} homeostasis (Portis and Heldt 1976). The level of Ca^{2+} in chloroplasts can rise upon illumination by light (Moore and Åkerman 1984; Miller and Sanders 1987; Kreimer et al. 1988) or after dark transition (Sai and Johnson 2002), and can follow a circadian rhythm (Johnson et al. 1995), but does not respond to mechanical stress or cold (van Der Luit et al. 1999). Within the chloroplast, Ca^{2+} is required for the electron flow at photosystem II (Kauss 1987), stabilizes the high redox potential form of cytochrome b-559 (McNamara and Gounaris 1995) and is an important co-factor and activity-regulator of enzymes like NAD kinase (Moore and Åkerman 1984). Indeed, the differential distribution of Ca^{2+} and Mg^{2+} between the stroma and thylakoid lumen during the dark and light phase has been shown to contribute to the regulation of the "on-off" state of chloroplasts (Ettinger et al. 1999).

A direct influence of the chloroplast on the cytoplasmic Ca^{2+} dynamics was revealed by the analysis of the Ca^{2+} -binding protein CAS (Ca^{2+} sensing receptor) (Han et al. 2003; Nomura et al. 2008; Weinl et al. 2008). CAS has originally been reported as an extracellular Ca^{2+}-sensing receptor, exhibting a high capacity to bind

calcium (10–12 calcium ions per molecule) (Han et al. 2003). However, several studies revealed that the protein is exlusively localized in chloroplasts (Nomura et al. 2008; Vainonen et al. 2008; Weinl et al. 2008). Within the chloroplast, CAS is targeted to the thylakoid membrane, and is light-dependent phosphorylated (Vainonen et al. 2008; Weinl et al. 2008). CAS knock-out plants exhibit retarded growth, however, activity of the photosystem is not affected in these plants (Vainonen et al. 2008). When grown under low Ca^{2+} conditions, CAS knock-down plants display delayed bolting and are not able to induce flowering (Han et al. 2003). Ca^{2+}-induced cytoplasmic Ca^{2+} release is impaired in CAS knock-down and knock-out plants and these lines are impaired in Ca^{2+}-induced stomatal closure responses (Han et al. 2003; Nomura et al. 2008; Weinl et al. 2008). However, CAS knock-out plants can respond to externally imposed Ca^{2+} oscillations and then display normal stomatal closure reactions, indicating that the ability to respond to cytoplasmic Ca^{2+} elevations in mutant plants is not affected. This points to a function of CAS in the generation of cytoplasmic Ca^{2+} transients that are required for stomatal closure (Weinl et al. 2008) and indicates that the chloroplast targeted Ca^{2+} sensor protein CAS somehow connects cytoplasmic and chloroplast Ca^{2+} dynamics. This function somewhat resembles that of the Ca^{2+} buffer protein CRT in the ER. Similarly, loss of CAS could lead to a reduced buffer capacity of the chloroplasts, implicating that less Ca^{2+} can be allocated from the chloroplasts to the transient cytoplasmic increase of Ca^{2+}. These recent findings surprisingly highlighted the interconnection and importance of chloroplasts for the cellular Ca^{2+} machinery.

6 Channels and Transporters shaping Ca^{2+} Signals

6.1 Influx of Ca^{2+}

Several different Ca^{2+} permeable channel activities were reported to exist at the plasma membrane of plants that can mediate the influx of Ca^{2+} into the cytosol and have the potential to modulate the cellular Ca^{2+} signature depending on their specific activation properties (White et al. 2002; Demidchik and Maathuis 2007). In general, Ca^{2+} permeable channels can be classified as voltage dependent and voltage independent/ligand dependent channels (White et al. 2002). Additionally, stretch activated calcium channels exist at the plasma membrane (Cosgrove and Hedrich 1991; Dutta and Robinson 2004; Nakagawa et al. 2007). These different channel types can co-exist in certain cell types, allowing the cells to respond to a wide range of signals and to differentiate between nutrient and signaling requirements (Miedema et al. 2001; Miedema et al. 2008). Variability in the specific abundance of the different channel types could contribute to the specific needs of a cell type or tissue (Demidchik et al. 2002). However, it should be noted that the molecular identification and characterization of true Ca^{2+} – specific channels from plants has still not been reported.

6.1.1 Voltage Dependent Channels

Voltage dependent channels are separated in to depolarization activated Ca^{2+} permeable channels (DACCs) and hyperpolarization activated Ca^{2+} permeable channels (HACCs) (White et al. 2002). Depolarization activated channels contribute to the short transient influx of Ca^{2+} during signal responses, since they enter a quiescent state at constant depolarized membrane voltage (Thion et al. 1998). Hyperpolarization activated channels exhibit a large Ca^{2+} conductance and could contribute to a sustained Ca^{2+} influx regulating signaling and nutrition in fast growing tissues or cell types (Miedema et al. 2001, 2008), but are inactivated by increased levels of intracellular Ca^{2+} (Hamilton et al. 2000). Consequently, both channel types could function interdependently. Hyperpolarization of the membrane could activate HAC channels. Influx of Ca^{2+} would inhibit HACC activity and depolarize the membrane which then would activate DAC channels (Hamilton et al. 2000; Miedema et al. 2001). In addition, voltage dependent K^+ channels could also contribute to the influx of Ca^{2+} (Fairley-Grenot and Assmann 1992; Wegner and De Boer 1997; White et al. 2002).

Plant annexins are small proteins capable of Ca^{2+}-dependent membrane binding and insertion and appear to create Ca^{2+} influx pathways especially during stress responses involving acidosis (Mortimer et al. 2008). Some annexins were recently shown to assemble into Ca^{2+}-permeable channels in the plasma membrane and endomembranes of plant cells, which could be activated by Ca^{2+}, hyperpolarization and ROS (Demidchik and Maathuis 2007; Mortimer et al. 2008). In addition, annexins from *Zea mays* were reported to create Ca^{2+} permeable transport pathways and to regulate cytoplasmic Ca^{2+} concentration (Laohavisit et al. 2009).

6.1.2 Ligand Gated Channels

While the molecular identity of voltage dependent channels is still of uncertainty, ligand gated channels are nonselective cation channels represented by Cyclic nucleotide gated channels (CNGCs) and Glutamate receptors, which are important for ion homeostasis of Ca^{2+} and different other cations like K^+, Na^+ and others (Hua et al. 2003a; Ali et al. 2006). Individual CNGCs harbor different selectivity filters, indicating that certain CNGCs exhibit distinct selectivity for cations (Kaplan et al. 2007). Indeed, tobacco plants overexpressing a CNGC gene were hypersensitive to Pb^{2+}, while Arabidopsis plants harboring a T-DNA insertion within the respective CNGC1 gene were more tolerant to Pb^{2+} (Arazi et al. 1999; Sunkar et al. 2000). In Arabidopsis 20 CNGC were identified which are expressed in different tissues of the plant (White et al. 2002). In general, CNGCs are activated by cAMP and cGMP and harbor a binding site for Calmodulin which partially overlaps with the binding domain for cNMPs. Therefore, binding of Ca^{2+}/Calmodulin results in inactivation of CNGCs, due to blocking of the cNMP binding domain (Hua et al. 2003b; Ali et al. 2006). Moreover, for CNGC2 it was also suggested that the channel is blocked by high external Ca^{2+} concentrations (Hua et al. 2003a). CNGC2 was originally

identified as the "defence no death" (dnd) 1 mutant, which fails to induce the Ca^{2+} mediated hypersensitive response to an avirulent strain of the pathogen *Pseudomonas syringae* and exhibits enhanced resistance to pathogens (Yu et al. 1998; Clough et al. 2000). Interestingly, mutants of CNGC2 are specifically hypersensitive to external Ca^{2+}, but have a normal Ca^{2+} content. Therefore, it was suggested that CNGC2 contributes to calcium signaling (Chan et al. 2003). In addition to CNGC2, CNGC4 (Balague et al. 2003), CNGC11 and CNGC12 (Yoshioka et al. 2006; Urquhart et al. 2007) were also implicated in mediating pathogen responses, while CNGC18 mediates tip growth of pollen (Frietsch et al. 2007).

Similar to CNGCs, Glutamate receptors (GLR) are also non-selective cation channels and 20 genes encoding GLRs were identified in Arabidopsis (White et al. 2002). GLRs are activated by Glutamate and Glycine as well as by other amino acids and mediate increases of cytosolic Ca^{2+} concentration (Qi et al. 2006). It is assumed that GLRs are important for plant Ca^{2+} nutrition (Demidchik and Maathuis 2007) but also have a role in calcium dependent photomorphogenesis. Application of GLR antagonists impaired plant light-signal transduction and resulted in enlarged hypocotyls and reduced chlorophyll accumulation (Lam et al. 1998; Brenner et al. 2000).

6.1.3 Vacuolar and ER Ca^{2+} Channels

Electrophysiological analyzes of the vacuolar membrane identified currents that are indicative for the function of voltage dependent channels and ligand gated channels. Among these, the "Two-pore channel" 1 (TPC1) appears to encode the depolarization activated slow vacuolar (SV) channel of the tonoplast (Hedrich and Neher 1987; Peiter et al. 2005). Plants lacking TPC1 are deficient in SV channel activity (Peiter et al. 2005). Remarkably, although the SV channel is the most abundant vacuolar channel, loss of SV channel function does not or only marginally impair calcium signaling events mediated by ABA or different biotic and abiotic factors which partially rely on influx of Ca^{2+} from intracellular stores (Peiter et al. 2005; Ranf et al. 2008). These observations suggest that SV-channels contribute only modestly to the modulation of cytoplasmic Ca^{2+} concentration by Ca^{2+} influx from the vacuole (Perez et al. 2008). Therefore, the exact functional role of TPC1 is still uncertain (Pottosin and Schonknecht 2007). Moreover, it needs to be considered that further not well characterized voltage dependent channels, that have been described as a fast vacuolar channel (FV) (Hedrich and Neher 1987) and as a Ca^{2+} insensitive vacuolar channel (CIVC) (Ranf et al. 2008), are likely to contribute to Ca^{2+} fluxes across the vacuolar membrane (Allen and Sanders 1994).

The identity and characterization of plant vacuolar ligand-gated Ca^{2+} channels is even less advanced than that of ligand-gated channels from the plasma membrane. By applying either caged compounds, or by direct patch clamp techniques, it was revealed that InsP3/InsP6 and cADPR mediated Ca^{2+} release, suggesting the existence of ligand-gated channels (Schumaker and Sze 1987; Alexandre 1990; Gilroy et al. 1990; Allen et al. 1995; Lemtiri-Chlieh et al. 2003). However,

considering the paucity of molecular data confirming the existence of such channels, it remains possible that these compounds may indirectly activate channels by binding to receptors which subsequently activate voltage dependent channels like SV and FV channels (Lemtiri-Chlieh et al. 2003).

Similarly, it was also suggested that the ER contributes to InsP3/InsP6 and cADPR mediated Ca^{2+} release (Muir and Sanders 1997; Martinec et al. 2000; Navazio et al. 2001). Besides these two types of channels, a unique ligand gated channel appears to exist at the ER which is activated by NAADP (Navazio et al. 2000).

6.2 *Efflux of Calcium*

After release of Ca^{2+} into the cytosol, Ca^{2+} is actively transported out of the cytoplasma against the electro-chemical gradient to restore the normal cytoplasmic Ca^{2+} level. This finally leads to the observed Ca^{2+} transient and it should be emphasized that a tight regulation of Ca^{2+} efflux is as equally important for Ca^{2+} signaling as the more intensively studied influx mechanisms.

Extrusion of calcium is achieved by P-type calcium-ATPases and by Ca^{2+}/H^{+} antiporter systems. While pumps mediate high-affinity low-turnover Ca^{2+} export, antiporter provoke low-affinity high-capacity export. Therefore, antiporter reduce the Ca^{2+} cytoplasmic level to a few micromolar after signal mediated influx of Ca^{2+}, while calcium-ATPases further lessen the cytoplasmic Ca^{2+} concentration to the resting level and maintain the Ca^{2+} homeostasis (Bush 1993; Hirschi 1999).

Ca^{2+} efflux transport activity appears to be coordinatively regulated with the influx of Ca^{2+} and specific regulation in response to defined stimuli has been reported (Bush et al. 1993; Gao et al. 2004). However, the underlying principles of Ca^{2+} efflux regulation are still poorly understood. Specific hormones can differentially activate the transporter systems of the ER or tonoplast. After a Ca^{2+} transient, Ca^{2+} released by GA seems to be mainly transported out of the cytoplasma via the ER transporters. In contrast, ABA activates transport activity at the ER and the tonoplast (Bush et al. 1993).

6.2.1 Calcium-Proton Antiporter

In the Arabidopsis genome 6 genes encode for putative Ca^{2+}/H^{+} antiporters, designated as cation exchangers (CAX) (Maser et al. 2001; Shigaki et al. 2006) that contribute to the regulation of Ca^{2+} (Catala et al. 2003; Cheng et al. 2003; Zhao et al. 2008). In addition, five cation calcium exchanger (CCX) proteins (also termed CAX7-11), related to K^{+} dependent Na^{+}/Ca^{2+} antiporters are encoded in the Arabidopsis genome (Shigaki et al. 2006). Moreover, four putative antiporters are encoded in the genome of Arabidopsis, which exhibit EF hand Ca^{2+} binding motifs suggesting that they are directly regulated by Ca^{2+} (Shigaki et al. 2006).

CAX proteins harbor a *N*-terminal regulatory/autoinhibitory domain, which binds to an adjacent region within the *N*-terminus (Pittman et al. 2002a; Mei et al. 2007). It has been observed that individual CAX proteins can have different transport capacities, metal selectivity and transcriptional regulation (Hirschi et al. 2000; Pittman et al. 2002b). Although individual CAX proteins can function specifically in distinct responses to definite stimuli (Zhao et al. 2008), CAX1 and CAX3 could also form functional heteromers (Cheng et al. 2005; Zhao et al. 2009). Additionally, different regulatory proteins could interact with CAX proteins to modulate their transport activity (Cheng and Hirschi 2003; Cheng et al. 2004a, b). CAX1-CAX4 are localized to the vacuole (Hirschi et al. 2000; Cheng et al. 2002a, 2003, 2005), but anti-porter activity was also reported to reside at the plasma membrane (Kasai and Muto 1990; Luo et al. 2005).

Several attempts were performed to change the cellular calcium levels of plants by overexpressing CAX proteins, either to improve plant tolerance against various stress regimes or to improve the availability of calcium for human nutrition. Overexpression of the truncated version (lacking the regulatory domain) of the vacuolar antiporter CAX1 from Arabidopsis in tobacco leads to an altered Ca^{2+} homeostasis. Although plants contained more total Ca^{2+}, plants showed Ca^{2+} deficiency symptoms. In accordance with this, plants also displayed hypersensitivity to Mg^{2+}, Na^{+} and to cold shock (Hirschi 1999). It was discussed that overexpression of AtCAX1 resulted in over-accumulation of Ca^{2+} in the vacuole and, therefore, by simultaneous reduction of the cytoplasmic Ca^{2+} concentration caused the deficiency symptoms.

6.2.2 Phosphorylated-type ATPases

Classical Ca^{2+} P-ATPases belong to the second subclass (II) of Phosphorylated(P)-type ATPases. Two different types of P_{II} ATPases are found in plants. P_{IIB} ATPases contain an autoinhibtory *N*-terminal region (autoinhibited calcium ATPases, ACAs; 10 members), which is absent in P_{IIA} type proteins (ER type calcium ATPases, ECA, 4 members) (Sze et al. 2000). The autoinhibitory domain in P_{IIB} type proteins can be relieved by the binding of Calmodulin, which results in activation of the pump (Harper et al. 1998). On the other hand, the activity of the P_{IIB} type Ca^{2+}-ATPase ACA2, is inhibited by phosphorylation within the *N*-terminal regulatory domain. Interestingly, this regulatory function is mediated by an another Ca^{2+} binding protein, a CDPK (Hwang et al. 2000). P_{IIA} type ATPases are found at the ER (ECA1) (Liang et al. 1997), the Golgi (ECA3) (Mills et al. 2008) and endosomes (also ECA 3) (Li et al. 2008). Besides being Ca^{2+} transporters, ECAs are also important for regulating the Mn^{2+} homeostasis of plants, transporting excess Mn^{2+} out of the cytoplasm (Wu et al. 2002; Li et al. 2008; Mills et al. 2008). The existence of ECAs at the Golgi and/or Endosomes could also be important for exocytotic processes as, for example, the vacuolar sorting receptor PV72 interacts with target proteins in a Ca^{2+} dependent manner (Shimada et al. 2002; Watanabe et al. 2002).

P_{IIB} type ATPases are found at the ER (ACA2) (Harper et al. 1998), the vacuole (ACA4, ACA11) (Geisler et al. 2000; Lee et al. 2007), the plasma membrane (ACA8, ACA9, ACA10) (Bonza et al. 2000; Schiott et al. 2004; George et al. 2008) and also at the plastid envelope (ACA1) (Huang et al. 1993). Transcript levels of ACAs are stress regulated (Carena et al. 2006). The importance of a P_{IIa} type Ca^{2+}-ATPase activity in regulating the cytoplasmic Ca^{2+} level is exemplified by an analysis of a Ca^{2+}-ATPase loss-of-function mutant in the moss *Physcomitrella patens*. While wildtype plants exhibit a transient Ca^{2+} release after applying Na^{+} stress, loss of function mutant lines exhibit a sustained elevation of Ca^{2+} (Qudeimat et al. 2008). Interestingly, the sustained increase of Ca^{2+} concentration impaired the expression of salt stress-induced genes and rendered mutant plants less tolerant to Na^{+} stress (Qudeimat et al. 2008), implicating a direct causal relation between the proper formation of a Ca^{2+} signature and stress tolerance. In Arabidopsis, analyses of loss-of-function mutations of ACA9 and ACA10 implicate these pumps in specific functions, like in pollen tube growth and in inflorescence development of plants, respectively (Schiott et al. 2004; George et al. 2008)

Moreover, several P_I type proteins, which are mainly heavy metal transporters are also implicated in Ca^{2+} transport. AtHMA1 (heavy metal ATPase), a heavy metal transporter supposed to function in detoxification processes for heavy metals is a P_I-ATPase that localizes to the chloroplast envelope. In addition to heavy metals like Cu^{2+}, AtHMA1 transports Ca^{2+} with high affinity and is specifically inhibited by thapsigargin like SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPase)-pumps from animals (Seigneurin-Berny et al. 2006; Moreno et al. 2008).

7 Signal Response Coupling of Calcium

Diverse stimuli specifically induce changes in cellular and apoplastic Ca^{2+} concentration (Cessna et al. 1998; Pauly et al. 2001; Lecourieux et al. 2002, 2005; Rentel and Knight 2004; Kosuta et al. 2008). The specific signatures of such Ca^{2+} transients can be unique to a defined cue but different stimuli can also induce similar Ca^{2+} responses. Typical examples of such Ca^{2+} signature are presented in Fig. 1c, and below we discuss only selected representative instances to illustrate this facet of Ca^{2+} signaling.

7.1 Differences in Salt and Mannitol Responses

Several reports showed that specific Ca^{2+} transients can be detected after salt or drought stress (imposed by mannitol application) in different cell types of the root, which, however, exhibited similar response signatures (Knight et al.

1997; Kiegle et al. 2000). Therefore, the existence of a Ca^{2+} independent factor was suggested that would allow to discriminate between the Ca^{2+} signatures evoked by drought and salt stress (Knight et al. 1997). In contrast, aleurone cells of barley responded differentially to either mannitol or sodium, leading to decrease and increase of cytosolic Ca^{2+}, respectively (Bush 1996). A detailed study by Gao et al. (2004) addressing the interconnection of cytoplasmic and apoplastic dynamics of Ca^{2+}concentration in roots of Arabidopsis provides new insights how Ca^{2+} could function as a specific signal in these different stress responses (Gao et al. 2004). Here, the Ca^{2+} elevation within the cytosol was simultaneously recorded to the changes of apoplastic Ca^{2+}. The response to mannitol resulted only in a minor transient cytoplasmic Ca^{2+} elevation. In contrast, sodium stress resulted in a transient cytoplasmic Ca^{2+} spike which was more pronounced than after mannitol application (Pauly et al. 2001; Gao et al. 2004). However, after this initial spike a slow but constant rise of cytoplasmic Ca^{2+} concentration was observed (see Fig. 1c) (Gao et al. 2004). Additionally, apoplastic Ca^{2+} elevation was also observed under sodium stress (Gao et al. 2004), which could result from enhanced exocytosis (Homann and Tester 1997; Belan et al. 1998). As sodium ions can exchange Ca^{2+} from the membrane or from cell wall components this apoplastic rise in Ca^{2+} concentration may contribute to membrane protection (Williams 1970; Hauser et al. 1976; Brett and Waldron 1996). An apoplastic calcium rise was not observed under drought stress, most likely since mannitol does not exchange calcium from membrane and cell wall components (Gao et al. 2004).

7.2 Differences in Symbiotic Calcium Responses

Symbiotic plants can interact with nitrogen-fixing rhizobial bacteria or with arbuscular mycorrhizal fungi that aid nutrient uptake of the plant. Although both signals (Nod factor, or mycorrhizal factors) require the same machinery for Ca^{2+} signaling, the outcome is quite different. While Nod factors induce nodulation, interaction with symbiotic fungi does not induce nodulation (Kosuta et al. 2008). Although both factors induce Ca^{2+} oscillations, their signatures are remarkably different. Initial, Ca^{2+} spiking triggered by Nod factor perception induces a subsequent large rise of cytoplasmic Ca^{2+} in regard of duration and amplitude. After a descent of cytoplasmic Ca^{2+} to basal levels, immediate and recurrent increases of calcium occur which finally result in repetitive and periodic spiking. This is in contrast to the oscillations induced by the mycorrhizal factor. Here, the transient is shorter and lower in amplitude (only 17% of the Nod factor spike). After the drop to basal level, there is a gap of different length of low Ca^{2+} before the next spike is recorded which can differ in duration and amplitude from the previous spike (see Fig. 1c) (Kosuta et al. 2008). These differences could result in the different observed symbiotic outcomes.

8 Calcium Binding Proteins

Different classes of Ca^{2+} binding proteins represent the cellular "currency" to translate Ca^{2+} signals into defined downstream response reactions. Here we will focus on three main classes of Ca^{2+} binding proteins, which all harbor EF hands for Ca^{2+} binding. These are the Calmodulins (CaMs) and their related Calmodulin like proteins (CMLs), the Calcium-dependent protein kinases (CDPKs) and the calcineurin-B like (CBL) proteins which form a network with CBL-interacting protein kinases (CIPKs).

8.1 Calmodulin

Calmodulins are small proteins of about 148 amino acids, composed of four Ca^{2+}-binding EF hands which are arranged in a dumbbell structure in that EF hands 1/2 and 3/4 are separated by a alpha-helical handle (Strynadka and James 1989). Upon Ca^{2+} binding, CaMs undergo a structural change, from a closed to an open conformation which enables binding to target proteins (Chin and Means 2000).

In Arabidopsis, 7 genes encode for only 4 Calmodulin isoforms because 3 Calmodulins share an identical amino acid sequence (CaMs 2, 3 and 5) (McCormack et al. 2005). Additionally, Arabidopsis contains 50 CaM-like proteins, which harbor variable numbers (between 2 and 6) of EF hands that could account for different Ca^{2+} binding affinities (McCormack and Braam 2003).

CaMs are generally cytoplasmic proteins, which can translocate into the nucleus or to cellular membranes upon binding to different target proteins (Deisseroth et al. 1998; Chung et al. 2000). Additionally, Ca^{2+} binding proteins related to Calmodulin can be secreted into the apoplastic space, potentially regulating cell wall regeneration (Sun et al. 1995) and the growth of pollen by affecting activity of hyperpolarization activated channels (Ma et al. 1999; Shang et al. 2005). CML18 has been reported to localize within the vacuole here regulating the function of the Na^+/H^+ antiporter NHX1 (Yamaguchi et al. 2005).

About 170 different target proteins of CaMs were identified up to now (Jun et al. 1996; Reddy et al. 2002; Yang and Poovaiah 2003; Popescu et al. 2007). CaMs/CMLs are important regulators of metabolism, cell structure proteins, proteins of the phyotohormone signal network, ion transporters, heat shock proteins and proteins regulating translation and transcription (Reddy et al. 2002; Du and Poovaiah 2005; Popescu et al. 2007; Du et al. 2009). CaM or CMLs are directly implicated in fine tuning the hypersensitivity response after pathogen infection. While NO production is mediated by CaMs or CMLs, like CML24 (Ma et al. 2008), the channel which is important for Ca^{2+} release, which then results in NO production, is also inhibited by CaMs (Ali et al. 2007). This could be important to prevent excess accumulation of Ca^{2+}, and would lead to the observed transient increase of Ca^{2+}. CaMs are also supposed to have an important role during

photomorphogenesis (Neuhaus et al. 1993) and a specific function in light dependent development of seedlings was assessed for CaM7 that can act as a transcriptional regulator by binding to Z- and G-box light responsive promoter elements (Kushwaha et al. 2008).

Different mechanisms contribute to the specificity of responses mediated by individual CaMs and CMLs. In the tobacco plant *Nicotiana plumbaginifolia*, individual CaMs are differentially transcriptionally regulated. While NpCaM1 is upregulated by wind and cold, the expression of NpCaM2 is unaffected (van Der Luit et al. 1999). In Arabidopsis, one CaM and different CMLs are induced by touch (Braam et al. 1997). Additionally, CML expression can be tissue specific and can be modulated by stress, hormones and light (McCormack et al. 2005; Magnan et al. 2008). Post-transcriptional regulation of protein stability by methylation or phosphorylation could also play an important role (Oh and Roberts 1990; Leclerc et al. 1999; Kushwaha et al. 2008). An additional layer of functional diversification results from differential target protein regulation by CaM/CMLs (Lee et al. 2000; Popescu et al. 2007). Remarkably, it has been reported that targets can be activated by one CaM/CML and reciprocally inactivated by another CaM/CML (Lee et al. 1997; Leclerc et al. 1999; Kushwaha et al. 2008).

8.2 CDPKs

Calcium dependent protein kinases (CDPKs) evolutionary arose by fusion of a *N*-terminal serine/threonine kinase with a *C*-terminal Calmodulin EF hand Ca^{2+} binding domain, separated by an autoinhibitory junction domain. Classical CDPKs contain four EF hands, while "CDPK-related kinases" (CRKs) harbor three or less functional EF hands (Harper et al. 2004).

The junction domain functions as a pseudosubstrate. When no Ca^{2+} is bound to the Calmodulin domain, the junction domain interacts with the kinase domain and blocks phosphorylation of target proteins. Upon Ca^{2+} binding, the Calmodulin domain binds the junction domain displacing it from the kinase domain and thereby leading to activation of the kinase (Hrabak et al. 2003).

In Arabidopsis 34 CDPKs are encoded in the genome. All CDPKs harbor a *N*-terminal "variable domain" upstream of the kinase domain, which can differ in sequence and length (Cheng et al. 2002b). The "variable domain" can determine CDPK localization and can represent a docking site for regulatory 14-3-3 proteins (Lu and Hrabak 2002). CDPKs are differentially localized, and have been found to reside in the cytoplasma and nucleoplasma, but are also associated with the cytoskeleton, plasma membrane, ER or peroxisomes (Putnam-Evans et al. 1989; Martin and Busconi 2000; Lu and Hrabak 2002; Dammann et al. 2003; Choi et al. 2005).

Regulation of CDPK kinase activity is achieved by a complex interplay of membrane translocation, phosho-lipid binding and auto- as well as trans-phosphorylation (Schaller et al. 1992; Farmer and Choi 1999; Szczegielniak et al. 2000,

2005). Some CDPKs are transcriptionally regulated (Hrabak et al. 2003) and could be regulated by their protein stability (Satterlee and Sussman 1998; Zhu et al. 2007). Since each CDPKs display distinct composition of various EF hands with different affinities to calcium, differential activation of CDPKs depending on the respective cellular Ca^{2+} concentration may occur. Certain CDPKs display a very low activation requirement suggesting that these kinases are already constitutively active at resting cytoplasmic Ca^{2+} levels (Lee et al. 1998).

Functional analysis of two CDPKs from Arabidopsis that are strongly expressed in guard cells (CPK3 and CPK6), revealed that loss-of-function mutants of CPK3 and/or CPK6 are impaired in Ca^{2+} and ABA-dependent activation of S-type anion channels (Schroeder and Hagiwara 1989; Mori et al. 2006) and in the ABA-dependent activation of Ca^{2+} channels (Hamilton et al. 2000; Pei et al. 2000; Mori et al. 2006). Consequently, these mutant plants exhibited reduced stomatal closure after application of ABA or after artificially imposing Ca^{2+} oscillations (Mori et al. 2006). However, long term stomatal closure in these mutants as well as ABA-mediated inhibition of seed germination were not affected, implicating that CPK3/CPK6 are specifically regulating the rapid stomatal closure (Mori et al. 2006). In contrast, loss of function of CPK4/CPK11, two highly related cytoplasmic/nucleoplasmic localized CDPKs, reduces the sensitivity of mutant plants to ABA in seedling germination and growth, and stomatal closure is partially impaired in response to ABA (Zhu et al. 2007). CPK4 and CPK11 interact and phosphorylate ABA-responsive transcription factors ABF1 and ABF4 in the presence of ABA (Uno et al. 2000; Zhu et al. 2007). However, ABFs also interact and are phosphorylated by other CDPKs, implicating further kinases in regulating ABA responses in Arabidopsis (Choi et al. 2005).

Phosphorylation of the transcriptional activator "Repression of shoot growth" (RSG) by CDPK1 from tobacco enables binding of 14-3-3 proteins and represses the function of RSG during GA responses (Ishida et al. 2008). CDPK2 from tobacco is transiently activated by phosphorylation specifically after pathogen infection (Romeis et al. 2000, 2001), and is triggering different stress response pathways within the cell (Ludwig et al. 2005). Furthermore, CDPKs are implicated in root development (Ivashuta et al. 2005), wound response (Szczegielniak et al. 2005), secretion and vacuolar function in response to GA (McCubbin et al. 2004), pollen tube growth and pollen tube polarity (Estruch et al. 1994; Yoon et al. 2006), response to salt, drought stress and potassium homeostasis (Saijo et al. 2000; Ma and Wu 2007). These findings illustrate the functional diversity of CDPKs in various biological processes.

8.3 CBLs and CIPKs

The third class of Ca^{2+} binding proteins is represented by the group of Calcineurin-B like (CBL) proteins (Kudla et al. 1999; Batistic and Kudla 2004). Similar to CaMs, CBLs contain four EF hands to bind Ca^{2+} (Nagae et al. 2003; Kolukisaoglu

et al. 2004; Sanchez-Barrena et al. 2005), but contain an unconventional first EF hand, which encompasses 14 aminoacids instead of 12 aminoacids typical for a canonical EF hand (Nagae et al. 2003). Nevertheless, this unique EF hand is still able to bind Ca^{2+} (Nagae et al. 2003; Sanchez-Barrena et al. 2005, 2007), and could play an important role in the selective interaction with CBL-partner proteins. In Arabidopsis 10 genes encode for CBL proteins (Kolukisaoglu et al. 2004). CBL1, CBL4, CBL5 and CBL9 are *N*-terminal myristoylated proteins (Ishitani et al. 2000; Batistic et al. 2008) that, in addition, harbor further cysteine residues in the vicinity of the myristoylated glycine suggesting further acylation. Indeed, CBL1 has been shown to undergo modification by palmitate or stearate, and together with the myristoyl modification, these lipid modifications are important for correct plasma membrane targeting and function of the CBL1 protein in stress response (Batistic et al. 2008). CBL2, CBL3, CBL6 and CBL10 lack a classical N-myristoylation site. Instead, these proteins harbor an extended *N*-terminal region that is important for correct sub-cellular targeting of the proteins (Batistic et al. 2009).

CBL proteins interact and regulate the activity of a certain class of protein kinases, designated as CBL-interacting protein kinases (CIPKs) (Shi et al. 1999). In Arabidopsis, 26 genes encode for CIPKs, which belong to the third subgroup of SNF-related protein kinases (SnRK3) (Hrabak et al. 2003; Batistic and Kudla 2009). It has been suggested that binding of CBLs to the CIPKs via the conserved NAF domain of these kinases (Albrecht et al. 2001) relieves autoinhibition of the kinase, which then results in kinase activation and target phosphorylation (Guo et al. 2001b; Fujii and Zhu 2009). Moreover, CIPKs can interact with PP2Cs (Ohta et al. 2003) and crystallization studies implicate that CIPK24 either interacts with CBLs or PP2Cs, excluding the formation of a trimeric complex (Sanchez-Barrena et al. 2007). Therefore, the on-off state of the CIPKs may be regulated by the interaction with CBLs (on state) or type 2C protein phosphatases (off state).

Several mechanisms contribute to generating signaling specificity within the CBL-CIPK network. Preferential complex formation between certain CBLs and CIPKs enable a focused signal transmission of signals from the calcium sensor proteins to the kinases (Albrecht et al. 2001). Additionally, certain pairs of CBL-CIPK complexes are localized at different cellular compartments, and are differentially expressed in different tissues or in response to stresses, thereby enabling spatial and temporal regulation of the network. For example, CBL4 is mainly expressed in roots, while CBL10 is mainly expressed in leaves (Kim et al. 2007). Both calcium sensor proteins can interact with CIPK24, which is expressed in both tissues. However, CBL4/CIPK24 complexes are localized at the plasma membrane while CBL10/CIPK24 complexes accumulate at the tonoplast thereby creating a dual functioning kinase (Fig. 1d). The alternative formation of CBL/CIPK24 complexes may enable simulatenous Ca^{2+}-dependent regulation of Na^+ extrusion in the root and Na^+ sequestration into the vacuole in the shoot of salt stressed plants (Kim et al. 2007). In general, CBL and CIPK proteins are critical for controlling the response to different stress situations like salt and osmotic stress (Albrecht et al. 2003; Cheong et al. 2003; D'Angelo et al. 2006; Tripathi et al. 2009), response to and regulation of ABA synthesis (Kim et al. 2003; Pandey et al. 2004), nitrate

homeostasis (Hu et al. 2009) root development (Tripathi et al. 2009) and stomatal movement (Cheong et al. 2007). CIPK11 negatively regulates the plasma membrane Arabidopsis H^+-ATPase 2 (AHA2), which mediates hyperpolarization of the plasma membrane (Fuglsang et al. 2007). CBL4 together with its interacting protein kinase CIPK24 form the specific "Salt overly sensitive" (SOS) pathway, which regulate the sodium/proton antiporter SOS1 at the plasma membrane (Fig. 1d). During salt stress, calcium influx is detected by CBL4, which activates CIPK24 and subsequently activates SOS1, to extrude excess sodium out of the cell (Halfter et al. 2000; Qiu et al. 2002; Quintero et al. 2002). CBL1 and CBL9 target CIPK23 to the plasma membrane to activate the potassium channel AKT1 to maintain K^+ homeostasis under low potassium conditions (Li et al. 2006; Xu et al. 2006; Cheong et al. 2007) (Fig. 1d). The identification of further targets fo CBL/CIPK complexes currently remains one of the main challenges to further our understanding of this complex signaling network.

9 Conclusions

Beginning in the middle of the last century plant biologists uncovered the crucial nutritional and structural role of Ca^{2+} for plants. However, in this regard Ca^{2+} never attracted as much attention as for example K^+. It was the surprising notion, that only a tiny fraction of the bio-available calcium, namely the free cytoplasmic Ca^{2+} pool and its regulated dynamics, modulates a plethora of biological processes that sparked an immense interest in this ion. Consequently, during the following decades of the last century an immense amount of observations accumulated that linked changes in cellular Ca^{2+} concentration and distribution to the regulation of many diverse processes of plant growth and development.

The extensive involvement of Ca^{2+} frequently leads to the vexing question: how can one ion specifically control so many events? Current research is beginning to provide answers. Ca^{2+} regulation in plants involves many facets that can define and adjust responses in both time and space. The unequal distribution of Ca^{2+} in the cell provides the basis for rapid Ca^{2+} fluxes and the resulting concentration changes. Influx channels on the plasma membrane and release channels from internal stores provide several ways to generate rapid ion elevations or to create local gradients. The frequency as well as amplitude modulation, provide means of generating signals that have unique properties. Once these signals are generated, then a wide variety of Ca^{2+}-decoding components interpret and relay these signals. Complex signaling networks, prominently involving CDPKs and CIPKs translate this information into phosphorylation events thereby simultaneously amplifying and specifying response reactions. It is an emerging picture that plants possess myriad ways in which Ca^{2+} can operate as the intermediary in transducing stimuli into the appropriate responses. The challenge for the near future lies in characterizing the underlying functional principles of signal response coupling and in identifying the prime targets of Ca^{2+} regulated phosphorylation events.

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