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The ER and Cell Calcium

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Abstract Calcium is one of the most versatile messengers in biological systems, translating developmental and environmental cues into cellular responses. The endoplasmic reticulum (ER) constitutes one of the most important calcium holding organelles in higher eukaryotes. Upon stimulation, calcium is released from the ER into the cytosol where it may trigger downstream effectors. However, the release of calcium may also affect internal ER functions, such as protein folding and secretion. It is therefore important not only to view cytosolic calcium signals as isolated events, but also in context to the organellar calcium status. In animals, the calcium levels of the ER can be sensed by other calcium resources, such as the plasma membrane, which may allow calcium uptake or release depending on the overall demand in the cell. In this chapter we have tried to convey the diverse aspects of calcium and its potential impact on different ER processes, and organellar communications, in plants.

1 Introduction

Plants are equipped with an intricate signalling network to interpret a constantly changing environment. This network constitutes a variety of components, ranging from proteins to ions, which trigger complex cellular responses. Calcium represents an important signalling molecule in all living systems (Sanders et al. 2002; Berridge et al. 2003). The plant cell sequesters calcium in different subcellular compartments, e.g. the vacuole, the cell wall, the chloroplasts, the mitochondria and the endoplasmic reticulum (ER). Upon stimulation, calcium may be released instantaneously from these compartments into the cytosol where it can coordinate downstream effectors. The calcium signalling network embraces so far approximately 700 proteins in the plant cell, creating a mosaic of potential interacting combinations (Reddy and Reddy 2004).

The resting level of free cytosolic calcium in a plant cell is approximately 100 nM, but may rapidly be increased to several mM during calcium influx (Sanders et al. 1999). Alterations in the spatiotemporal progression of the calcium influx may result in distinct Ca^{2+}-signalling signatures, i.e. variations in amplitude, duration and frequency of the signal (McAinsh et al. 1995; Allen

and Schroeder 2001). Although the calcium influx is necessary for the signal to progress, a sustained elevation of cytosolic calcium may pose a potential threat to the cell. High cytosolic calcium levels may cause energy-containing phosphate molecules, e.g. adenosine triphosphate (ATP), to precipitate, a process which may be lethal to the cell (Bush 1993). A tight coordination of the Ca^{2+}-signalling network is therefore of great importance to the cell.

A variety of abiotic stimuli, e.g. light (Johnson et al. 1995; Love et al. 2004), temperature variations (Catala et al. 2003; Kim et al. 2003), touch (Knight et al. 1991; Haley et al. 1995), gravity (Plieth and Trewavas 2002), osmotic and oxidative stress (Ng et al. 2001; Coelho et al. 2002), as well as biotic stimuli, e.g. hormones (Schroeder et al. 2001; Murata et al. 2001), fungal elicitors (Klusener et al. 2002), pathogens (Romeis et al. 2001) and nodulation factors (Levy et al. 2004), utilize calcium as a signalling factor. Although these disparate cues all trigger calcium releases, the cell's ultimate responses reflect the stimulus. Accordingly, the calcium paradox becomes apparent; How does the cell know which stimulus to react on, and how is a distinct response achieved? Several plausible and combinatorial theories have been presented: 1) Other signalling molecules may work in conjunction with calcium to trigger specific downstream effectors, 2) Depending on the primary messenger(s), different subcellular calcium-stores may be triggered, 3) The calcium signal may

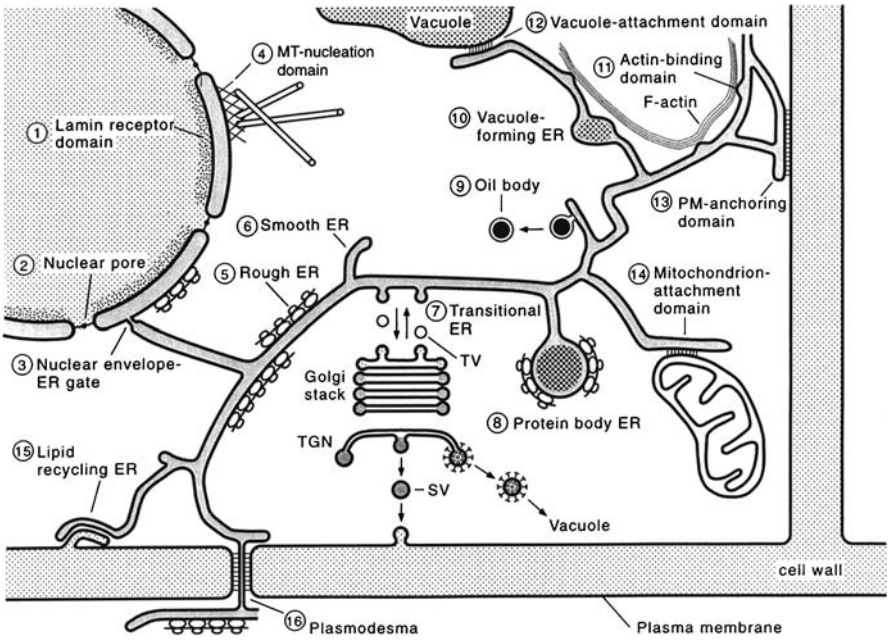

Fig. 1 Schematic model of different ER subcompartments in a plant cell. Reproduced from Staehelin (1997) with permission

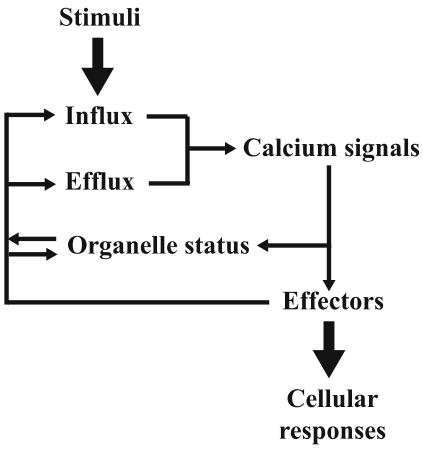

Fig. 2 Flow-chart of stimuli-induced Ca^{2+}-signalling network. Stimuli may activate calcium discharge from Ca^{2+}-holding compartments. The release of calcium affects downstream effectors and organellar processes. The effectors and the organellar status may in turn regulate the progression of the signalling pattern, i.e. the shape and size of the signal. The calcium signal is thus tightly monitored through a feedback network. Modified and extended from Sanders et al. (2002)

contain cues in itself, i.e. variations in amplitudes and frequencies of the signal, 4) The downstream effectors may differ depending on the developmental stage and type of cell that is triggered, 5) A sub-compartmentalization of calcium-release and uptake devices may generate calcium-denser areas in the cell. The latter provides an interesting aspect regarding the ER. At least 16 ER subcompartments have been described in plant cells (Fig. 1; Staehelin 1997). Several of these are in close proximity to other calcium-holding organelles and may therefore participate in calcium cross-talk between different stores.

Although calcium is well established as a cytosolic signalling source, the effects of calcium fluctuations on internal organellar processes are largely unexplored. Calcium fluxes over the ER membrane in animal cells may affect processes such as protein folding, secretion and glycosylation status. A dynamic view, incorporating cytosolic as well as organellar effects, of cellular calcium fluctuations may therefore be crucial to understand how calcium affects the physiology of the plant cell (Fig. 2).

2 Components

A reductionist approach to comprehend the ER calcium network requires the identification of its basic components. The ER in a typical plant cell contains Ca^{2+}-permeable channels, triggered by a variety of agonists, Ca^{2+}-pumps

Table 1 The major ER calcium network components in animals and plants

Components	Inhibitors	Activators	Physiological implications
Calcium permeable channels			
Animal:			
IP 3 -receptor (3)	High Ca^{2+}, CaM[1]	IP_3, Low Ca^{2+}, ATP, NADH, Phosphorylation[1]	Oozyte fertilization, Neuronal signalling[1]
Ryanodine- receptor (3)	High Ca^{2+}, Mg^{2+}, NO, CaM[2]	Ryanodine, cADPR, low Ca^{2+}, ATP, NO, CaM, Clq[2]	Mono- and divalent ion permeable[2]
Plant:			
IP_3-receptor (?)	—	IP_3[3]	—
Ryanodine-receptor (cADPR) (?)	Ruthenium red, 8-NH_2-cADPR[3]	cADPR[3]	—
NAADP- receptor (?)	Gd^{3+}, Ruthenium red[3]	NAADP[3]	—
LCC1- channel (?)	La^{3+}, Gd^{3+}, Erythrosin B[3]	Voltage-gated[3]	Ca^{2+}-, Ba^{2+}-, Sr^{2+}-permeable, Voltage-gated[3]
BCC1- channel (?)	Gd^{3+}, Cu^{2+}, Zn^{2+}, pH, H_2O_2[3]	Voltage-gated[3]	Ca^{2+}-, Ba^{2+}-, Sr^{2+}-permeable, Voltage-gated, Mechano-sensitive?[3]
Calcium efflux transporters			
Animal:			
Type IIA:			
SERCA1 (2)	Thapsigargin[4,5]		Brody's disease, Reduced Ca^{2+}-uptake[4,5]
SERCA2 (2)	Phospholamban, Sarcolipin, Thapsigargin[4,5]		Darier disease, Cardiac arrestment, Reduced Ca^{2+}-stores[4,5]
SERCA3 (5)	Thapsigargin[4,5]		Affect glucose homeostasis in pancreas, Altered Ca^{2+}-homeostsis in pancreas and aortic smooth muscle[4,5]

Table 1 continued

Components	Inhibitors	Activators	Physiological implications
Plant:			
Type IIA:			
ECA1	CPA, Vanadate, Erythrosin B[6]	—	Ca^{2+}- and Mn^{2+}-transport, Sensitivity to external Ca^{2+}-depletion, Cytoskeletal maintenance?[6]
Type IIB:			
ACA2	Vanadate, Erythrosin B, Phosphorylation[6]	CaM[6]	Ca^{2+}-transport[6]
Calcium binding proteins			
Animal:			
Calreticulin (2)	—	—	Chaperone, Ca^{2+}-homeostasis, Cardiac development, SERCA2B regulator, Cell adhesion, Immunology[7]
Calnexin/Calmegin (2)	—	—	Chaperone, Ca^{2+}-homeostasis, Fertility, SERCA2B regulator[8]
Calsequstrin (2)	Phosphorylation[9]	—	Ca^{2+}-homeostasis, Ryanodine-R regulator, Cardiac development, thiredoxin-related[9]
Plant:			
Calreticulin (3)	—	—	Chaperone, Ca^{2+}-storage, Sensitivity to external calcium depletion[10,11]
Calnexin (2)	—	—	Chaperone[10]

References: [1] Patterson et al., 2004; [2] Fill and Copello, 2002; [3] White, 2000; [4] Prasad et al., 2004; [5] Wuytack et al., 2002; [6] Sze et al., 2000; [7] Gelebart et al., 2005; [8] Michalak et al., 2002; [9] Beard et al., 2004; [10] Vitale and Denecke, (1999); [11] Persson et al., 2001 and specific references within these. Numbers in parenthesis indicate number of isoforms.

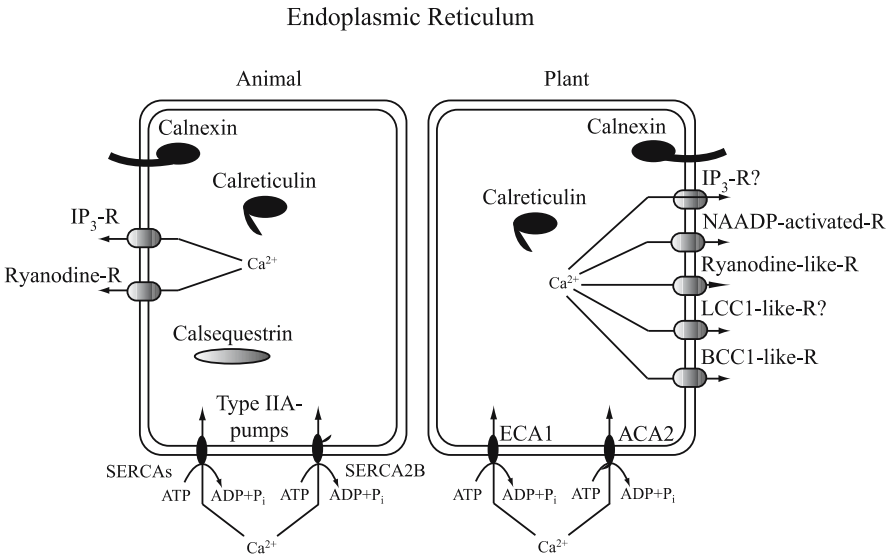

Fig. 3 Comparative representation of components of the ER calcium network in animals and plants. IP_3-R: Inositol 1,4,5-triphosphate-receptor, NAADP-R: Nicotinic acid adenine dinucleotide phosphate-receptor, BCC1: Bryonia calcium channel 1, LCC1: Lepidium calcium channel 1, ATP/ADP: adenosine triphosphate/diphosphate, SERCA: SR/ER calcium ATPase

and Ca^{2+}-binding proteins (Crofts and Denecke 1998; Sanders et al. 2002). These components were largely discovered and analyzed through biochemical efforts (for review see Bush 1993). Several of the components have subsequently been more thoroughly characterized, and placed into a cellular context, through multidisciplinary approaches (for review see Sanders et al. 2002). Data mining of the Arabidopsis and rice genomes have further increased the number of components connected to the ER calcium regulatory network in plants (Baxter et al. 2003; Persson et al. 2003). More recently, comparative analyses of calcium components in plants and animals have, not surprisingly, provided evidence for overlapping as well as diverging processes among the two kingdoms (Fig. 3, Table 1; Berridge et al. 2003; Reddy and Reddy 2004).

2.1 Calcium Permeable Channels

While the free cytosolic calcium level is kept at submicromolar levels, the total calcium concentration in the ER/Sarcoplasmic reticulum (SR) has been estimated to be 5–10 mM in animal cells (Meldolesi and Pozzan 1998). A similar level is also expected in typical plant ER (Sanders et al. 2002). The steep calcium gradient over the membrane may facilitate an instant discharge of

calcium from different organelles into the cytosol in response to agonists (Berridge et al. 2003). The calcium influx is mediated through ion channels, referred to as calcium permeable channels (Fig. 4; White et al. 2000). In plants, calcium channels have been detected in the ER (Klusener et al. 1995, 1999), the plasma membrane (PM), the tonoplast, the nucleus and the plastid envelope (for review see White 2000). The channels are classified according to the mode of activation, i.e. voltage-gated, ligand-activated, cytoskeletal connectivity and/or membrane stretch activation.

ER-localized calcium channels have only recently begun to get characterized in plants (Table 1; Klusener et al. 1995, 1997, 1999). The first ER channel to be identified was purified from touch-sensitive tendrils of the dicot plant *Bryonia dioica* and was referred to as BCC1 (Klusener et al. 1995, 1997). This voltage-gated channel was analyzed through lipid bilayer techniques and exhibited a high calcium conductance at low pH (Klusener et al. 1995, 1997). Inhibition studies further showed that BCC1 is sensitive to Gd^{3+}, H_2O_2, Cu^{2+} and Zn^{2+}.

Another putative ER calcium permeable channel (LCC1) was purified from garden cress (*Lepidium sativum* L.); Klusener et al. 1999). Characterization via lipid bilayer techniques suggested that the channel is voltage gated and strongly rectifying. The LCC1 channel further showed a simpler gating mechanism for calcium release than BCC1 (Klusener et al. 1999). LCC1 may be inhibited by both erythrosine B and the trivalent cations La^{3+} and Gd^{3+}. Although some similarities are evident between the two channels, the kinetic and pharmacological properties are distinctly different (Klusener et al. 1999). These observations suggest that the two channels are not simply orthologs from two different plant species. However, whereas the BCC1 appeared to

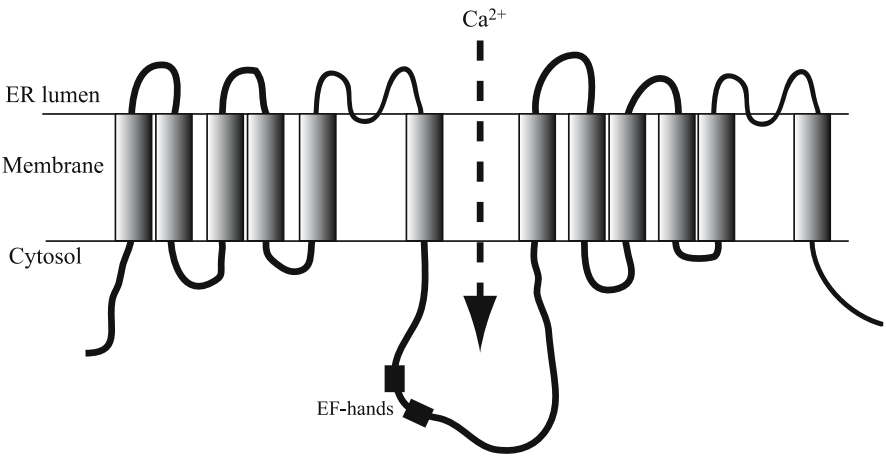

Fig. 4 Schematic topological model of a PM voltage-gated Ca^{2+}-permeable channel (TPC1). Putative EF-binding hands are indicated

be highly enriched in ER membrane fractions, an alternative location of the LCC1 is plausible (Klusener et al. 1999).

Additional calcium permeable channels have also been implicated for the plant ER (Table 1; Muir and Sanders 1997; Navazio et al. 2000, 2001). Radiolabelled calcium was used to show that influx of calcium could be mediated by applying either nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic-ADP-ribose (cADPR) or inositol-1,4,5-triphosphate (IP_3), all potent calcium elicitors in animal cells. NAADP may trigger intracellular Ca^{2+} releases in animal cells (for review see Patel 2004). An NAADP-sensitive Ca^{2+}-store was detected in ER-enriched membrane fractions from cauliflower (Navazio et al. 2000). The channel was independent of the cytosolic level of free calcium, and thus not responsible for calcium induced calcium releases (Navazio et al. 2000). Surprisingly, the Ca^{2+}-release was insensitive to typical L-type channel antagonists, i.e. verapamil and diltiazem.

In contrast to the novelty of NAADP-sensitive calcium stores in plants, cADPR-sensitive stores have been detected in the tonoplasts (Allen et al. 1995). A potential non-vacuolar cADPR-releasing activity was, furthermore, detected in ER-enriched fractions from cauliflower (Navazio et al. 2001). Analogous to the vacuolar cADPR-activated Ca^{2+}-release, the ER-associated release was fully inhibited by ruthenium red and 8-NH_2-cADPR, a specific antagonist for cADPR-gated channels in animal cells (Navazio et al. 2001). IP_3 is the most prevalent Ca^{2+}-releasing agonist for the ER/SR in animal cells (Berridge et al. 2003), and IP_3-triggered Ca^{2+}-releases have been observed from tonoplasts in plants (Allen et al. 1995). However, using microsomal fractions from cauliflower, Muir and Sanders (1997) demonstrated a non-vacuolar IP_3-sensitive calcium store. IP_3 may furthermore bind ER-enriched membrane fractions from *Chenpodium rubrum*, suggesting a potential IP_3-triggered calcium pool in the ER (Martinec et al. 2000).

The diversity of ER Ca^{2+}-releasing agonists further emphasizes the importance of the organelle in different intracellular Ca^{2+}-signalling events. However, similar to the identification of LCC1, the localization of the cADPR-, IP_3- and NAADP-releasing sources, i.e. the ER, in plants were achieved by enriching membrane fractions through sucrose gradient separation (Muir and Sanders 1997; Navazio et al. 2000, 2001). The NAADP-releasing source in animal cells appears, however, to be clearly separated from the ER/SR compartment (for review see Patel 2004). Identification of the genes encoding ER localized calcium channels may therefore provide further evidences to the subcellular localization of the cADPR-, IP_3- and NAADP-sensitive calcium stores.

2.2 Calcium Efflux Transporters

The activity of a variety of Ca^{2+}-transporters are coordinated with the calcium channels to tune the frequencies and amplitudes of the progressing

calcium signal and are used to restore resting cytosolic calcium levels following the signalling event. Two major classes of Ca^{2+}-efflux transporters exist in plants: Ca^{2+}/H^{+}-antiporters and Ca^{2+}-pumps. The Ca^{2+}/H^{+}-antiporters are driven by a proton gradient (Hirschi 1999), whereas the Ca^{2+}-pumps (Ca^{2+}-ATPases) are directly coupled to ATP hydrolysis as an energy source (Sanders et al. 2002).

The Ca^{2+}-pumps and Ca^{2+}/H^{+}-antiporters have distinctly different kinetic properties. Whilst the antiporters have low affinity, but high transporting capacity for calcium (Pittman and Hirschi 2003), the pumps exhibit opposite properties (Sze et al. 2000). These characteristics suggest distinct complementary roles in the calcium efflux process, where the low affinity antiporters rapidly remove the majority of cytosolic calcium and the high affinity pumps fine-tune the basal cytosolic calcium levels. These complementary activities make sense in subcellular locations in which both efflux systems reside, such as the vacuole and the PM (Hirschi 1999; Kasai and Muto 1990; Sanders et al. 2002). Antiporters are, however, not expected to function in the ER, since the proton gradient over the ER membrane is believed to be too weak to drive proton-coupled antiporters. Thus, the dynamics of Ca^{2+} transport into the ER are controlled exclusively by Ca^{2+}-ATPases, providing a potentially important distinction from the multiple transporters systems operating at vacuoles or PM.

A compelling difference between calcium transport into the plant and animal ER is that plants utilize two types (IIA and IIB) of Ca^{2+}-ATPases instead of one (Table 1; Sze et al. 2000; Geisler et al. 2000). The type IIA pumps are found in the ER of both plants and animals. Due to their subcellular location, these pumps are referred to as ER-type calcium pumps. The type IIA pumps are not stimulated by calmodulin (CaM), but are inhibited by cyclopiazonic acid (CPA) and thapsigargin, two well-characterized inhibitors of animal ER/SR Ca^{2+}-ATPases. In contrast to animals, the plant ER is also equipped with a calmodulin-stimulated pump that belongs to the type IIB family. In animals, the type IIB pumps are instead exclusively localized to the PM. Because of their initial discovery in the PM of animal cells, they are referred to as PM-type Ca^{2+}-ATPases (PMCA). Since the type IIB type pumps are not restricted to a single membrane system in plants, they are referred to as ACAs (Autoinhibited Ca^{2+}-ATPases). In contrast to the type IIA (ER-type) pumps, the type IIB (ACA-type) pumps are stimulated by CaM, and are relatively insensitive to CPA and thapsigargin. Members of both type IIA and B pump families are clearly distinguished by sequence homologies and appear to have emerged before the separation of the plant and animal kingdoms (Sze et al. 2000; Baxter et al. 2003). The presence of two distinct types of Ca^{2+}-ATPases further illustrates the complexities in regulating calcium transport into the plant ER.

The Arabidopsis genome contains fourteen genes encoding Ca^{2+}-pumps (Sze et al. 2000; Baxter et al. 2003). Four genes belong to the type IIA (ER-type) family and are referred to as ECAs (ER-type Ca ATPases). There is

immunological and membrane fractionation evidence that ECA1 is most abundant in the ER, providing corroborating evidence that at least some members of this plant family are ER localized. The remaining 10 genes belong to the type IIB (ACA-type) family. Similar to ECA1, immunology and microsomal fractionation strongly indicates that ACA2 is most abundant in the ER. However, other members of the plant ACA family have been found to reside in the vacuole or PM. Thus, the CaM-regulated ACAs may be used in multiple subcellular locations. Of the 10 ACAs in Arabidopsis, a total of 3 isoforms are closely related to ACA2 and are expected to be ER localized (Baxter et al. 2003).

The mechanism of CaM activation of type IIB pumps has been examined for both plant (Sze et al. 2000) and animal isoforms (Clapham 1995). The animal type IIB pumps contain an autoinhibitor and CaM binding sequence located in the C-terminal domain. However, the plant ACAs have their autoinhibitor and CaM binding sequence at the opposite end of the protein, i.e. N-terminal domain (Fig. 5; Malmström et al. 1997; Harper et al. 1998; Baxter et al. 2003). The model for CaM-activation involves interaction of CaM to a site overlapping or juxtaposed to the autoinhibitor in the pumps. This binding event somehow disengages the autoinhibitor. A mutation that removes this N-terminal regulatory domain results in a constitutively active pump (Harper et al. 1998).

A useful tool for dissecting biochemical properties of both ACA and ECA Ca^{2+}-pumps has emerged through the use of heterologous expression in the K616 mutant strain of the yeast *Saccharomyes cerevisiae*. In the mutant, a deletion of two Ca^{2+}-pumps; PMR1 in the Golgi and PMC1 in the vacuolar membrane, result in a yeast strain that requires high levels (> 1 mM) of calcium for normal growth (Sze et al. 2000). The growth defect on calcium-depleted media appears to result from the inability to load the ER secretory system with sufficient amounts of calcium. The phenotype was complemented by the expression of the plant ECA1. Biochemical analyses of ECA1 expressed in the K616 yeast system furthermore confirmed that the activity of ECA1 was CPA sensitive, and that the pump was not stimulated by CaM (Liang et al. 1997; Liang and Sze 1998). In addition, the yeast system provided evidence that ECA1 has a dual capacity to transport both Ca^{2+} and Mn^{2+} (Wu et al. 2002). A biological function for Mn^{2+} transport by ECA1 is supported by the observation that a deletion of *ECA1* in Arabidopsis exhibits an increased sensitivity to Mn^{2+} toxicity.

In contrast to ECA1, ACA2 did not rescue the growth of yeast K616 on calcium-depleted media (Harper et al. 1998). However, the pump was able to complement the yeast strain when the N-terminal autoinhibitory domain was truncated. Using this observation as the foundation for a mutant screen, seven random intragenic mutations were identified that allowed ACA2 to complement the yeast strain (Curran et al. 2000). Three of these mutations were unexpectedly found in the stalk that connects the ATPase and trans-

membrane domains, a region outside the N-terminal autoinhibitory domain and not previously recognized as having regulatory functions. These new mutations implicate the stalk region as an important point of control for ATPase activity, possibly through a direct interaction with the autoinhibitor (Fig. 5). An analogous stalk mutation engineered into an animal PMCA-pump sug-

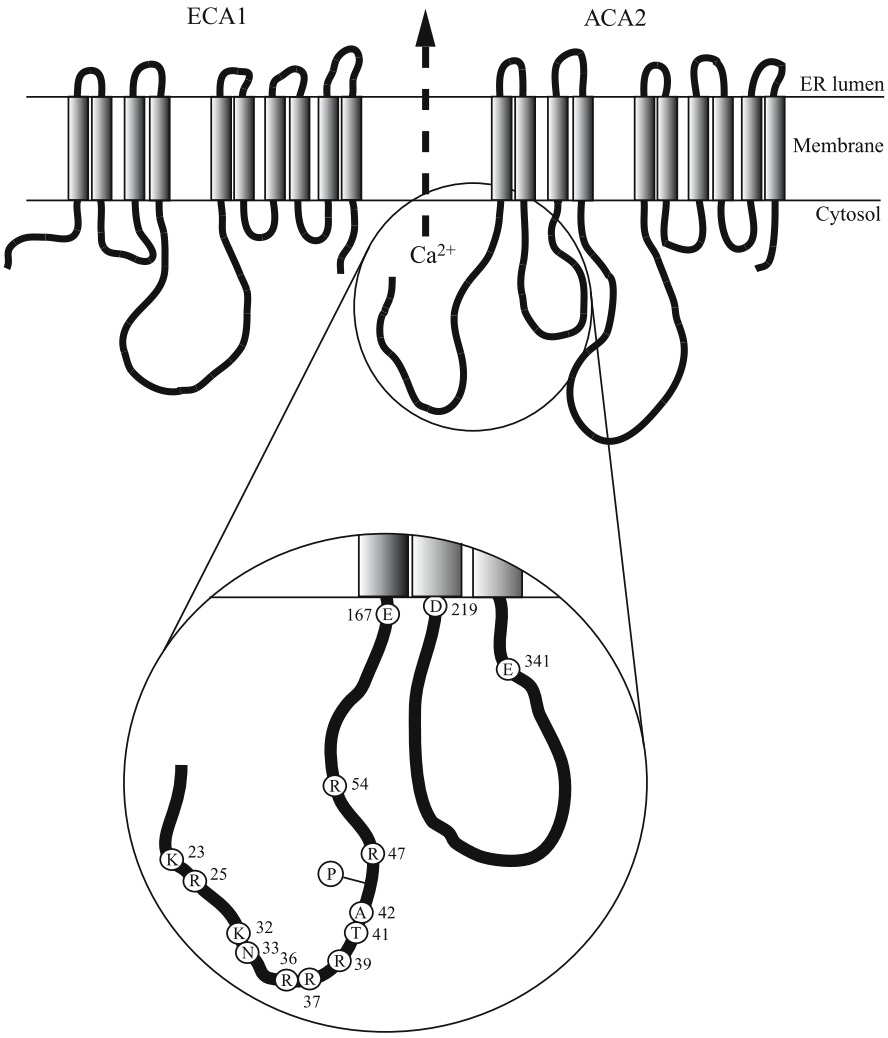

Fig. 5 Schematic topological models of the ER Ca^{2+}-ATPases, ECA1 and ACA2, in plants. The N-terminal region in ACA2 is enlarged to show amino acids involved in the autoinhibition of the ATPase (The corresponding numbers of the amino acids are indicated). The inhibitory phosphorylation site (Ser^{45}) on ACA2 is indicated with a **P**

gests that this region has general importance for regulation of all members of the type IIB pumps (Bredeston and Adamo 2004).

The distinct and redundant functions of the ECA and ACA pumps in the plant ER have yet to be delineated. However, the observation that ACA2 is CaM-activated identifies CaM as part of a feed-back regulatory pathway that controls the calcium dynamics associated with an ER calcium release. In addition, ACA2 may be inhibited by a separate calcium signalling pathway involving a plant-specific calcium-dependent protein kinase (CDPK; Hwang et al. 2000). Thus, the presence of both an ACA and ECA in the plant ER strongly suggests that plants carefully regulate the ER calcium efflux, and that this regulation is directly connected to feed back control by calcium signalling pathways.

2.3 Calcium-binding Proteins

The Ca^{2+}-holding capacity of different organelles is crucial for the calcium signalling system to perform efficiently. Maintenance of Ca^{2+}-buffering devices inside the organelles may therefore be essential to the cell (Crofts and Denecke 2001; Corbett and Michalak 2000). The ER/SR in animal cells contains a variety of calcium-binding proteins, e.g. calreticulin (Crt), calnexin (Cnx) and calsequestrin (Clq) (Corbett and Michalak 2000). These proteins may perturb both the calcium levels, as well as the influx/efflux efficiency, of the ER/SR (Camacho and Lechleiter 1995; Arnaudeau et al. 2002). Several homologs to these proteins have also been identified in higher plants (Fig. 6, Table 1; Vitale and Denecke 1999). In addition, other plant compartments, e.g. the vacuole and cell wall, also appear to hold Ca^{2+}-binding proteins (for review see Hirschi 2004).

The two major ER Ca^{2+}-binding proteins are Crt and Clq in animal cells (Table 1; Michalak et al. 1999). Whereas plant cells do contain Crt homologs (Vitale and Denecke 1999), no closely related Clq homologs have been identified. Crt is an ER luminal Ca^{2+}-binding chaperone, which holds three distinct domains; An N-terminal N domain with conserved histidine residues, a P do-

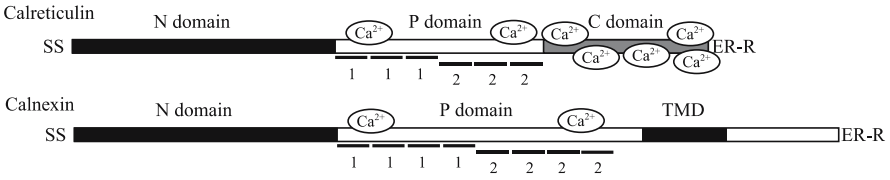

Fig. 6 Schematic representation of the two Ca^{2+}-binding proteins Crt and Cnx. Approximate regions for the N, P and C domains are indicated. Two repeat segments in the P domain are indicated with **1** and **2**, respectively. ER-R: ER retention signal, SS: Signal sequence, TMD: Transmembrane domain

main with low capacity but high affinity Ca^{2+}-binding properties and a C domain with high capacity but low affinity Ca^{2+}-binding properties (Fig. 6; Michalak et al. 1999). The protein can bind approximately 40 moles calcium per mol protein, mainly facilitated through the high capacity binding C domain (Baksh and Michalak 1991). Hence, the ER calcium content may depend on the Crt protein levels. Several studies in animal cells have also shown that overexpression of Crt leads to both an increase in the Ca^{2+}-signalling pool, and in free calcium levels, of the ER (Mery et al. 1996; Arnaudeau et al. 2002). Although no direct evidences for Crt as an effector of cytosolic calcium signalling exists in plants, Crt has been shown to be a potential effector of the ER calcium holding capacity and to protect against changes in external calcium levels (Persson et al. 2001; Wyatt et al. 2002; Åkesson et al. 2005).

Structural analyses of the central P domain of a rat Crt revealed an extended hairpin structure, comprised of three antiparallel β-sheets (Ellgaard et al. 2001). A similar structural feature is also evident in Crt's membrane bound homolog Cnx (Schrag et al. 2001). A putative calcium-binding site was indicated between Asp437, Asp118 and Ser75 in Cnx and was suggested as having a conformational role rather than directly affecting ligand-binding characteristics (Schrag et al. 2001). The tight coordination of the bound calcium to the P-domain of Crt/Cnx also implies constant maintenance of the bound calcium, even during prolonged depletion of the ER calcium (Schrag et al. 2001).

Whereas two Crt isoforms are present in animals (Persson et al. 2002), higher plants contain three Crt isoforms; Crt1a, Crt1b and Crt3 (Persson et al. 2003). While Crt1a and b show high sequence similarity (86% identity), Crt3 is distinctly different in several aspects. For example, both Crt1a and b contain higher levels of negatively charged amino acids in their C domains compared to Crt3 (approximately 35 and 25%, respectively). The high amount of negatively-charged amino acids in the C-domain is suggested to provide the main Ca^{2+}-binding characteristics for the protein (Baksh and Michalak 1991). However, no study has provided evidences for a correlation between the Ca^{2+}-holding potential of Crt and the amount of negatively-charged amino acids. A comparison between the three Crt isoforms in higher plants should therefore be relevant to resolve the Ca^{2+}-holding capacity of Crt.

Preliminary large-scale microarray data analyses have revealed that Crt1a and b are expressed in a very similar manner over approximately 1500 microarray datasets (Nottingham Arabidopsis Stock Center (NASC); Thelin and Persson, unpublished data). Several other ER chaperones, e.g. protein disulfide isomerase (PDI)-related homologs, also show a high degree of coexpression with Crt1a and b, indicating presence of an interactive chaperone matrix in the ER. Crt3, on the other hand, did not show coexpression with any known chaperone-like proteins, but instead exhibited coexpression with an iron-binding oxygenase family member and several pathogen-related proteins (Thelin and Persson, unpublished data). This further underscores a func-

tional dichotomy for the Crt family (Persson et al. 2003), and may provide insight to the physiological context of different Crts.

3 Orchestration of the Components

Identification and characterization of the components of the ER calcium network have provided important information to how these components may be regulated individually. A tight coordination of these components is, however, necessary to regulate both calcium fluxes, i.e. the amplitude and frequency of the cytosolic signal, as well as the internal ER calcium level. The challenge therefore lies in how these components act together, and affect each other, to achieve this regulation. While several aspects of how such coordination affects the calcium status of the ER/SR are becoming resolved in animal cells, analogous information is still largely lacking in plants.

The basic regulatory function is achieved through transcriptional coordination of the various components. This may be facilitated by the level of luminal ER calcium level, which works as a transcriptional switch for several components of the ER calcium network in animal cells (Waser et al. 1997). Both SERCA3 and Crt expressions are induced in response to depletion of ER calcium levels, e.g. by treatments with the ER Ca^{2+}-pump inhibitor thapsigargin or the calcium ionophore A23187, implying an attempt of the cell to increase the Ca^{2+}-holding potential of the ER (Waser et al. 1997; Liu et al. 2002).

The ER luminal proteins Cnx and Crt may coordinate the ER calcium dynamics in animal cells, i.e. influx, efflux and holding potential (Camacho and Lechleiter 1995; Michalak et al. 1999; Arnaudeau et al. 2002). Cnx and Crt can modulate the uptake of calcium by direct interactions with the Ca^{2+}-pump SERCA2B (Camacho and Lechleiter 1995; Roderick et al. 2000). In contrast to other SERCAs which consist of ten transmembrane segments, SERCA2B has eleven transmembrane regions (Bayle et al. 1995). Consequently, the carboxy terminus of the protein is located within the ER lumen. Crt may directly interact with the luminal carboxy terminus of SERCA2B, a characteristic possibly regulated by the glycosylation status of the pump (John et al. 1998). Coexpression of Crt and SERCA2B in *Xenopus* oocytes revealed that Crt effectively inhibits IP_3-mediated calcium oscillations, resulting in a sustained elevation of cytosolic calcium (John et al. 1998). In addition, Cnx may also affect SERCA2B activity, possibly through a direct interaction with the pump (Roderick et al. 2000). Analogous to Crt, coexpression of Cnx and SERCA2B in *Xenopus* oocytes caused sustained elevation of cytosolic calcium, generated through inhibition of SERCA2B. The inhibition was promoted by phosphorylation of a serine residue in the cytosolic carboxy-terminal of Cnx (Roderick et al. 2000). The two chaperones Cnx and Crt may thus both affect the ER

Ca^{2+}-uptake, coordinated through either internal ER processes or through cytosolic phosphorylation events.

Plants lack a SERCA2B homolog with a C-terminal luminal extension, and a direct effect on Ca^{2+}-fluxes by any luminal ER Ca^{2+}-binding proteins remains to be demonstrated. However, the activity of the Ca^{2+}-pump ACA2 may be regulated by phosphorylation (Hwang et al. 2000). The phosphorylation occurs in the cytosolic N-terminal region and inhibited the activity of the pump (Fig. 5). As described above, CaM may activate ACA2 by interacting with the autoinhibitory N-terminal domain of the pump. When CaM was bound to the pump, the inhibitory phosphorylation was prevented (Hwang et al. 2000). The "signalling" dynamics of the cytosol may thus orchestrate an activation or inhibition of ACA2. The phosphorylation may be mediated through a CDPK referred to as CPK1 (Hwang et al. 2000). So far only one CDPK, CDPK2, has been shown to localize to the ER (Lu and Hrabak 2002). Whether this CDPK phosphorylates ACA2 in vivo has not yet been tested.

Crt may also be post-translationally modified through phosphorylation and glycosylation (Baldan et al. 1996; Li et al. 2003; Persson et al. 2003). In vitro studies suggest that the phosphorylation is mediated through a casein kinase II/CK2 (Baldan et al. 1996). Whether this kinase can enter the ER lumen remains undetermined. Although the glycosylation status of animal Crts is suggested to mediate a cellular redistribution of the protein, no physiological functions for either glycosylation nor phosphorylation are so far apparent for plant Crts.

4 Cellular and Physiological Implications

The ability to coordinate the Ca^{2+}-signalling network, i.e. to achieve specific spatiotemporal characteristics of the signal, is of great importance for an agonist-stimulated signal to progress properly. Altering the abundance of different Ca^{2+}-signalling components may consequently skew the calcium signal and hence affect different sets of downstream targets. These targets may range from signal transmitters to signal effectors (Sanders et al. 2002). The signal transmitters, such as transcription factors and CaM-like proteins, may undergo conformational changes in response to Ca^{2+}-fluxes, which alter their binding ability to target molecules. In contrast, the signal effectors, such as various kinases and phosphatases, may directly trigger a covalent modification of their target molecules.

A major breakthrough in the visualization of calcium signals has been the development of calcium sensitive probes (Knight et al. 1991; Miyawaki et al. 1997). The most widely used probes are aequorins and cameleons. Whereas the aequorins are useful when measuring calcium changes in whole tissues

or a cell population, the cameleons can be used to measure Ca^{2+}-oscillations on a single-cell level. The cameleon construct consists of a recombinant protein based on a CaM flanked by two fluorescent proteins (Fig. 7A), which can be transformed into any organism and targeted to different organelles. When the calcium levels increase the CaM backbone undergoes a conformational change which brings the two fluorescent proteins in close proximity to each other (Fig. 7A; Miyawaki et al. 1997). By choosing fluorescent protein-parts with different excitation and emission wavelengths, the energy from one of the fluorescent protein-parts may be transferred to the other, referred to as fluorescent resonance energy transmission (FRET).

Combining cameleons with reverse genetic and molecular approaches has begun to increase our understanding for how different components affect the cellular calcium status in plants (Allen et al. 1999, 2000). This approach was used to dissect how a vacuolar H^+-ATPase affects Ca^{2+}-signalling in guard cells (Allen et al. 2000). By introducing cameleons into a line with a mutated vacuolar H^+-ATPase (*det3*), Allen et al. (2000) showed that the spatiotemporal calcium patterns generated by different stimuli changed in the mutant line compared to wild-type, and resulted in altered physiological responses of the guard cell (Fig. 7B,C; Allen et al. 2000). This report provided the first genetic evidence that distinct stimulus-specific calcium oscillations may facilitate specific physiological responses in plants.

The only component in the plant ER calcium network that has been investigated so far using genetic approaches is the Ca^{2+}-pump ECA1 (Wu et al. 2002). Disruption of the *ECA1* gene did not result in any visible phenotype when grown on standard Murashige Skoog growth medium (Wu et al. 2002). However, when grown on either low calcium (0.2 mM) or high manganese (0.5 mM) levels, seedlings grew very poorly (Fig. 8A). This may be due to an inhibition of cell expansion or cell division (Wu et al. 2002), similar to what has been reported for the yeast *pmr1* mutant, lacking a Golgi Ca^{2+}/Mn^{2+}-pump (Durr et al. 1998). In addition, the *pmr1* mutant showed defects in protein sorting and glycosylation processes, indicating a link between calcium and internal ER processes. Perturbing the cellular calcium homeostasis in plants by deletion of *ECA1* related Ca^{2+}-pumps may also affect intracellular organization (Adamikova et al. 2004). Disruption of an *ECA1* homolog in the fungus *Ustilago maydis* resulted in randomization of the microtubule network due to a sustained elevation of cytosolic calcium (Adamikova et al. 2004).

Overexpression of *CRT* also results in a conditional growth phenotype (Persson et al. 2001). Arabidopsis seedlings expressing a maize Crt1a/b homolog showed higher resistant to low levels of calcium in the growth medium (Fig. 8B). The increase in *CRT* expression further mediated a higher Ca^{2+}-holding potential of the ER in vitro (Persson et al. 2001). In addition, Arabidopsis plants expressing GFP fused to the high capacity Ca^{2+}-binding C domain of Crt showed an overall increase in cellular calcium (Wyatt et al. 2002).

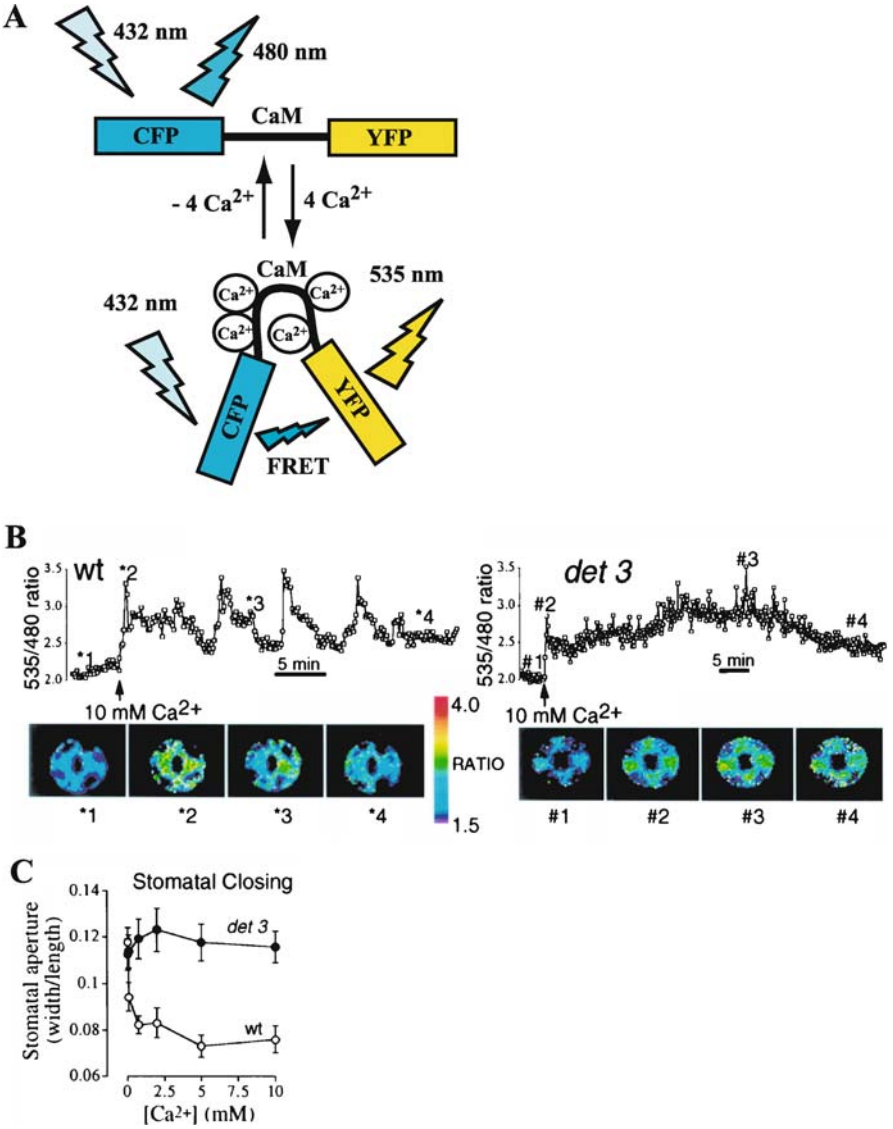

Fig. 7 Schematic model of the Ca^{2+}-indicator cameleon and its utilization in measuring defective Ca^{2+}-oscillations in the *det3* mutant. **A** The cameleon molecule, and its activation, consisting of a calmodulin (CaM) backbone fused to two fluorescent proteins. Modified from Miyawaki et al. (1997). **B** Application of external calcium evokes differences in cytosolic calcium responses in guard cells from wild-type and *det3* mutants. Intracellular Ca^{2+}-oscillations were induced applying 10 mM external calcium. Whereas the wild-type exhibited oscillatory Ca^{2+}-signals, Ca^{2+}-oscillations were abolished in the *det3* mutant. **C** The increase in external calcium caused stomatal closure in wild-type but not in the *det3* mutant. Reproduced from Allen et al. (2000) with permission. YFP: *Yellow* fluorescent protein, CFP: *Cyan* fluorescent protein, FRET: Fluorescent Resonance Energy Transfer

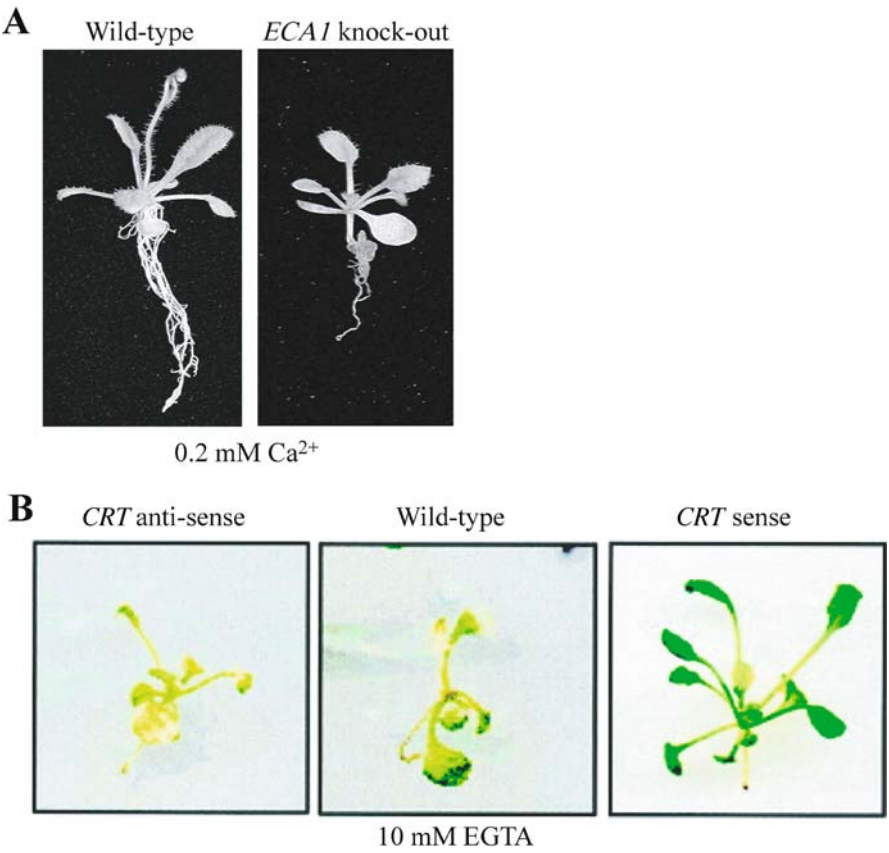

Fig. 8 Conditional growth phenotypes exhibited in *ECA1* knock-out plants and seedlings over expressing *CRT*. **A** Deletion of the *ECA1* gene results in delayed growth on medium containing reduced levels of calcium. Seedlings (5-day-old) transferred from MS medium to medium containing reduced calcium and grown for 10 days. **B** Correlation of *CRT* expression and seedlings ability to maintain growth on calcium-depleted medium. Seedlings (19 day-old) transferred from MS medium to medium containing reduced calcium (10 mM EGTA) and grown for 9 days. Reproduced from Wu et al. (2002) and Persson et al. (2001) with permission

The unique and redundant functions of different calcium stores in plant cells are not fully understood. However, calcium signals originating from the ER are expected to affect localized and global events, e.g. regulation of ER-generated calcium signatures and transcriptional events. The components decoding these calcium signals include CDPKs, CaM, as well as many of the other approximately 400 proteins in plants that have defined calcium binding EF-hands (Reddy and Reddy 2004). Of the 34 identified CDPKs in Arabidopsis (Harper and Harmon 2004), only AtCPK2 has been shown to localize to the cytosolic surface of the ER (Lu and Hrabak 2002). There is experimental

evidence that this association is dependent on myristoylation at the N terminus of AtCPK2. While the in vivo target substrates of CPK2 have yet to be determined, CDPKs may phosphorylate the ER calcium pump ACA2 (Hwang et al. 2000). In this case, phosphorylation is predicted to slow the rate of calcium efflux back into the ER, thereby possibly increasing the duration and magnitude of a calcium signal in the micro-environment around the ER. Since CDPKs are thought to be multifunctional kinases (Harper et al. 2004), many ER surface proteins are expected to be regulated by calcium signals decoded by isoform CPK2.

While investigations of how altered levels of Ca^{2+}-signalling components affect physiological responses have been initiated in plants, potential cross-talk between different Ca^{2+}-containing organelles are still largely unexplored. The ER calcium status in animal cells is tightly linked to Ca^{2+}-channels at the PM (Randriamampita and Tsien 1993; Fasolato et al. 1993; Putney et al. 1999). Consequently, depletion of the ER calcium stores activates the PM Ca^{2+}-channels and allows for refilling of the ER stores. This feedback process is referred to as capacitative calcium entry (Putney et al. 2001). The presently favoured model for capacitative calcium entry is a direct communication between PM Ca^{2+}-channels and the cADPR activated ryanodine receptors in the ER (Putney et al. 2001). The tight monitoring of the ER calcium status further strengthens the notion that the calcium ion is essential for proper maintenance of a variety of ER functions.

The ER calcium fluxes in animal cells also appear to influence the calcium status of the mitochondria (Rizzuto et al. 1993). This may be facilitated via the "hot-spot" hypothesis, in which IP_3-activated receptors in the ER are enriched at ER/mitochondria interfaces (Rizzuto et al. 2004). When calcium is released through the receptors, mitochondrial Ca^{2+}-uniporters facilitate uptake of calcium into the mitochondria. The increase in mitochondrial calcium may activate ATP production, but could also alter the organelle structure and trigger a release of apoptosis-activating substrates (Rizzuto et al. 2004).

Potential organellar cross-talk in the plant cell may, however, offer clues to phenotypic behaviours generated by genetic and molecular procedures. As discussed above, lower expression of either *CRT* or *ECA1* results in reduced growth on medium containing low levels of calcium (Fig. 8; Persson et al. 2001; Wu et al. 2002). These phenotypes were largely attributed to an overall decrease in ER calcium. However, overexpression of the vacuolar Ca^{2+}/H^+-antiport, *CAX1*, in tobacco displayed severe symptoms of calcium deficiency and contained a two-fold increase in cell calcium (Hirschi 1999). The phenotype could be reversed by adding exogenous calcium. Thus, a decrease in expression of at least two ER calcium network components, and an increase in expression of a vacuolar calcium network component, causes similar phenotypes. The explanation for this deceptive contradiction may lie in the distribution of calcium between the two organelles. If we assume that the protein level of a specific calcium handling protein is directly related to its

activity, then the decrease in both Crt and ECA1 should result in lower ER calcium levels. Similarly, increasing the *CAX1* expression may increase the vacuolar calcium efflux efficiency and therefore reduce the levels of accessible calcium for other organelles, e.g. the ER. Introducing cytosolic- and ER-targeted cameleons in crossed combinations of Crt, ECA1 and CAX1 plants should certainly present useful tools for visualization of potential cross-talk between the vacuole and the ER.

5
Calcium and Internal ER Regulation

It is well established that calcium works as an important cytosolic signal. In addition, the levels of calcium may be a potent switch for internal processes in various oganelles (Corbett and Michalak 2000; Rizzuto et al. 2004). The release and subsequent uptake of calcium during signalling events, creates continuous fluctuations of the available calcium in the organelle (Yu and Hinkle 2000). In animal cells, changes in the free ER calcium levels may control a variety of processes, e.g. protein synthesis and secretion (Corbett and Michalak 2000). Consequently in animal cells several ER chaperones, i.e. folding mediators, are regulated by calcium (Corbett et al. 2000; Frickel et al. 2002). Both Crt and Cnx interact with their substrates in a Ca^{2+}-dependent manner in animal cells. In vitro studies show that the lectin-like properties of these chaperones are significantly reduced when the calcium concentrations drop to levels observed in Ca^{2+}-depleted ER (Vassilakos et al. 1998). Furthermore, the latter unifies the dual functions of these proteins in the ER lumen; i.e. the chaperone and calcium-regulatory characteristics.

Expression of recombinant Crt proteins in a *CRT* knock-out mouse cell background, further emphasized the intimate link between the chaperone and calcium-regulatory aspects of Crt (Nakamura et al. 2001). Cells expressing *CRT* lacking its major Ca^{2+}-binding C-terminal domain exhibited a substantial decrease in the bradykinin-sensitive Ca^{2+}-signalling pool. The agonist bradykinin binds to a PM localized receptor protein and triggers an internal IP_3-induced ER calcium release. However, when measuring the binding of bradykinin to the receptor, Nakamura et al. (2001) discovered that the binding was severely impaired compared to control cells. The observed decrease in the ER calcium signalling may thus be explained by a misfolded bradykinin receptor.

Crt and several PDI-related proteins interact with each other in a Ca^{2+}-dependent manner in animal cells (Corbett et al. 1999). Interactions have been observed between PDI and Crt, which mainly seem facilitated by the high calcium capacity binding C domain in Crt and are reversibly increasing when the ER calcium levels are low. The interaction between Crt and PDI inhibits the isomerase activity, and thus the chaperone activity, of PDI

(Baksh et al. 1995). Crt can also interact with the PDI related protein ERp57. The initial interaction between Crt and ERp57 is independent of calcium concentration. However, the conformation of ERp57 may be indirectly altered through Crt at higher levels of calcium. This change presumably results in a tighter interaction between the two chaperones and the substrate. In addition, the substrate binding of Crt is reduced at low levels of calcium suggesting a lower chaperone efficiency in ER Ca^{2+}-depleted cells.

Additional chaperones and protein processors may also be affected by changes in the ER calcium levels. The binding protein BiP binds calcium and possesses a lower chaperone activity at low calcium levels in animal cells (Lievremont et al. 1997). Several other steps during protein synthesis, glycoprotein maturation and secretion are also impaired during lower calcium levels. The lower efficiency during these conditions may be reflected in a lower mode of protein–protein interactions.

Although very little is known about internal ER processes and calcium in plants, several of the affected components are present in plant cells. The model plant Arabidopsis contains two Cnx and three Crt isoforms (Persson et al. 2003). In addition, four PDI and two ERp57 homologs are also expressed in Arabidopsis (Persson et al. 2005). The interactive sites between ERp57 and Crt in animals are conserved in all of the plant homologs (Svensson and Persson, unpublished data), indicating that interactions may take place. Interestingly, whereas Crt and Cnx are conserved in most higher eukaryotes, the PDI protein family is extremely diverse (Persson et al. 2005). A comparative network scheme of interacting ER proteins from different species may therefore provide important evolutionary clues to the conservation and propagation of the ER chaperone matrix.

6 Perspectives

In plants, as expected by analogy to well studied animal systems, the ER is emerging as an important Ca^{2+}-source for intracellular signalling. Several important signalling components have been identified and characterized (Table 1; Sanders et al. 2002). However, approaches to reveal how these components are functionally orchestrated will be necessary to understand calcium related functions within the organelle, as well as cytosolic signalling events. Comparable studies in animal systems have revealed the ER, although a physical continuum, as a diverse and heterogenous organelle (Papp et al. 2003).

The construction and distribution of Arabidopsis insertion lines have provided the plant scientific community with a powerful tool to explore functional implications of genes of interest. The first gene involved in ER Ca^{2+}-signalling to be investigated using this approach was the ECA1 Ca^{2+}-

ATPase (Wu et al. 2002). Disruption of the *ECA1* gene resulted in reduced growth on medium with lower calcium contents. A similar growth defect was evident when an antisense construct of Crt was introduced into Arabidopsis plants (Persson et al. 2001). Combining molecular and genetic tools may therefore be a fruitful approach to assess the effects of altered ER calcium on plant growth. Crossing individual insertion lines may further reveal the extent of redundancy and functional overlap among different genes. Subsequent introduction of cameleons would then allow for a direct connection between the disruption/overexpression of genes and changes in calcium signatures.

Several additional approaches may be utilized to uncover the dynamics of the plant ER calcium network: 1) Comparable distribution of components using either fluorescently-labelled proteins or immuno-techniques, 2) Introduction of compatible fluorescently-labelled components for FRET analyses, 3) Large-scale microarray data mining to expose coregulatory networks of genes, 4) Assessment of protein–protein interactions through various proteomic efforts. These techniques should provide spatio-temporal information about how different components work in concert with each other under various conditions.

The emerging view in animal cells is that different organelles may sense and respond to the status of other organelles. This view has not been explored for Ca^{2+}-signalling and holding compartments in plants. The cross-talk in animal systems between, for example, the ER, PM and mitochondria has generated information to how changes in Ca^{2+}-homeostasis may affect the cellular status, e.g. apoptosis, protein synthesis and secretion. Given that the vacuole is the main Ca^{2+}-holding source in plant, a disruption of the calcium equilibrium between this organelle and the ER may provide insight into how the cellular distribution of calcium is facilitated.

Whilst most of the suggested approaches here refer to a macroscopic view of the ER and cellular Ca^{2+}-signalling, it is equally important to uncover structural information of the different enzymes. The structure of the P domain of Crt was solved using NMR and revealed a extended hairpin with three anti-parallel beta-sheets (Schrag et al. 2001). This information provided useful clues to how Crt interacts with other Ca^{2+}-regulated chaperones, such as ERp57 (Frickel et al. 2002). The structure of SERCA1a has similarly provided important information for functional aspects of Ca^{2+}-pumps (Toyoshima et al. 2004). These data may also reveal evolutionary relationships which may not be evident solely from sequence homology.

In this chapter we have tried to high-light the versatile aspects of calcium and its impact on a variety of ER processes. The tight coordination of cytosolic and organellar calcium contents emphasizes the importance of the ion in different compartments. The Ca^{2+}-fluxes should therefore simultaneously be viewed as a regulatory switch for cytosolic as well as internal organellar processes.

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