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Biochimica et Biophysica Acta 1763 (2006) 1209–1215

BIOCHIMICA ET BIOPHYSICA ACTA

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Review

Calcium signaling in plant cell organelles delimited by a double membrane

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Received 6 July 2006; received in revised form 13 September 2006; accepted 15 September 2006
Available online 20 September 2006

Abstract

Increases in the concentration of free calcium in the cytosol are one of the general events that relay an external stimulus to the internal cellular machinery and allow eukaryotic organisms, including plants, to mount a specific biological response. Different lines of evidence have shown that other intracellular organelles contribute to the regulation of free calcium homeostasis in the cytosol. The vacuoles, the endoplasmic reticulum and the cell wall constitute storage compartments for mobilizable calcium. In contrast, the role of organelles surrounded by a double membrane (e.g. mitochondria, chloroplasts and nuclei) is more complex. Here, we review experimental data showing that these organelles harbor calcium-dependent biological processes. Mitochondria, chloroplasts as well as nuclei are equipped to generate calcium signal on their own. Changes in free calcium in a given organelle may also favor the relocalization of proteins and regulatory components and therefore have a profound influence on the integrated functioning of the cell. Studying, in time and space, the dynamics of different components of calcium signaling pathway will certainly give clues to understand the extraordinary flexibility of plants to respond to stimuli and mount adaptive responses. The availability of technical and biological resources should allow breaking new grounds by unveiling the contribution of signaling networks in integrative plant biology.

Keywords: Calcium; Plant cell signaling; Plant cell organization; Cell compartmentation; Dynamics of cytosolic and organelle calcium

1. Introduction

Plants take up calcium from the soil by root hairs and transport the cation through the vascular system to the sink organs (leaves, flowers and fruits) using the driving force generated by evapotranspiration [1,2]. In the soil, calcium is generally present at concentrations high enough to prevent calcium deficiency in plants. However, bad redistributions of calcium from older tissues to developing ones lead to the so-called physiological disorders like bitter pit of apples and blossom end rot of watermelon. These costly disorders in horticulture have drawn the attention of plant physiologists who are trying to control and improve calcium uptake and redistribution [1,2]. Calcium is essential also for cell wall stability and expansion and exerts beneficial effects on plant vigor and fruit firmness. Besides its effects as a macro and structural element, calcium is fully recognized as a signal molecule [1–5]. An increase in free calcium concentration is one of the general events that relay an external stimulus to the internal cellular machinery to mount a biological response. As such, calcium is a second messenger that encodes changes in biotic and/or abiotic environmental parameters. Decoding information conveyed by calcium should allow the cell to generate an adaptive biological response. In this context, the total amounts of calcium are not the most important factor. Rather, it is the dynamic changes in free calcium in the cytosol and/or active cellular organelles that are translated into changes

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doi:10.1016/j.bbamcr.2006.09.024

in metabolism, growth, development and/or adaptation. Understanding how a simple and ubiquitous nutrient like calcium is implicated in a plethora of biological processes is an active field of research in eukaryotic cells and organisms including plants [1–6]. Due to its pleiotropic effects, a possibility is that calcium acts as a general switch responding indistinctly to a large array of stimuli. As a consequence, the specificity of the biological outcome would depend strictly upon a complex network of downstream signaling and amplification systems [7]. Indeed, examples suggesting that Ca^{2+} is a simple switch have been described and critically discussed. However, not all calcium signals act as a simple binary on–off switch in plants [7]. Rather, as established in animal models [6], changes in free calcium do not proceed in a stereotypical manner in plant cells but according to a signature depending upon the characteristics of the stimulus including its intensity, duration, timing and subcellular localization [5,8,9]. The calcium signature triggers downstream events in an ordered manner and manipulating a particular signature will modify the biological outputs of an initial stimulus.

Authoritative reviews on calcium signaling in plants are published and updated periodically with the refining of the conceptual frame and the methodological approaches [3–5,10]. Most of these reviews are concentrated on the regulation of changes in cytosolic calcium $[Ca^{2+}]_{cyt}$ and their effects on the functioning of plant cells. Indeed, the two biggest organelles in size e.g. the vacuole and the cell wall may be considered both as sources and buffering compartments of calcium mobilized to or expelled from the cytosol to prevent formation of insoluble salts (e.g. calcium phosphate) that are deleterious to cell integrity. Chloroplasts, mitochondria and nuclei contain also mM total calcium mainly sequestrated as bound calcium [8]. Consequently, these organelles surrounded by a double membrane constituting either a continuous barrier (chloroplasts and mitochondria) or punctuated by pores (nuclei) have to take up calcium from outside and may also contribute to regulate homeostasis of Ca^{2+} in the cytosol. Moreover, calcium-dependent events take place in chloroplasts, mitochondria and nuclei and, consequently, free calcium concentrations might be also regulated in these organelles, and might, by some manner contribute to specify cellular calcium signature. This short review aims at drawing attention to the spatial component of calcium signaling in plant cell organelles (essentially mitochondria, chloroplasts and nuclei) as part of the calcium toolkit.

2. Isolated plant organelles harbor calcium-dependent biochemical processes and are able to take up calcium from their internal environment

For the sake of clarity, we will consider successively the chloroplast, the mitochondrion and the plant cell nucleus.

2.1. Calcium movements and biological effects in isolated and in cellular chloroplasts

In vitro measurements of enzyme activities in the chloroplastidic stroma have shown that NAD kinase which provides NADP from NAD^+ for photosynthetic reduction to NADPH, is activated by Ca^{2+} [11]. Interestingly, illumination of isolated chloroplasts results in the uptake of Ca^{2+} from the incubation medium and leads to the decrease in the NAD^+ levels whereas inhibition of the light-induced Ca^{2+} uptake by ruthenium red reduces the light-dependent decrease in NAD^+ levels in the stroma [11].

The mechanism by which Ca^{2+} enters chloroplasts during illumination was a matter of controversy. A first line of evidence suggests that a H^+/Ca^{2+}-antiport process fueled by ATP [12] is responsible of Ca^{2+} uptake whereas other experimental data claim that the driving force is most probably due to changes in membrane potentials [11]. Recent data have established that both processes exist in the chloroplast and that they are specifically located on distinct plastidic compartments.

Thus, the initial rate of Ca^{2+} uptake across inner-envelope vesicles of pea chloroplasts is greater in right-side-out than in outside-out vesicles [13]. In right-side out vesicles, the uptake is stimulated by a pH gradient (high pH inside) or by a potassium diffusion gradient (inside negative) in the absence of a pH gradient. In either condition, addition of valinomycin in the presence of K^+, which dissipates the membrane potential gradient ($\Delta\psi$) but leaves the magnitude of the pH gradient unchanged, inhibits calcium uptake. These data suggest that the transport of Ca^{2+} is unidirectional via a potential-stimulated transport process at the inner- envelope membrane.

Ca^{2+} transported to the thylakoid lumen must cross the envelope membranes, the stroma and the thylakoid membrane [14]. Data obtained with isolated thylakoid membranes show that transthylakoid Ca^{2+} transport is stimulated by light or, in the dark, by adding ATP in the incubation medium. Addition of an H^+-translocating uncoupler which dissipates the thylakoid proton gradient generated by ATP hydrolysis inhibits Ca^{2+} transport. The dependence of the reaction on ΔpH suggests that a Ca^{2+}/H^+ antiporter localizes to the thylakoids.

Therefore, two distinct transport systems with different spatial localization allow Ca^{2+} to cross the inner-envelope membrane and the thylakoids. The coordinated functioning of the two systems allows the control of Ca^{2+} entry into the stroma and the thylakoid and regulates calcium-dependent processes. Ca^{2+} accumulated in the light may be bound in the stroma or sequestered in the thylakoids. The thylakoid transporter would contribute to remove excess Ca^{2+} from the stroma, in the light, otherwise high levels of free Ca^{2+} would inhibit carbon dioxide fixation.

In fact, the dynamics of changes in free Ca^{2+} levels in the chloroplast is more complex than expected by measurements in isolated chloroplasts or membrane preparation thereof. Using transgenic *Nicotiana plumbaginifolia* seedlings harboring the calcium bioluminescent reporter protein targeted to the chloroplast stroma, it has been shown that darkness provokes a large Ca^{2+} flux in the stroma [15]. The increase starts a few minutes after lights off, proceeds maximally within 20 min and returns to the background levels. The magnitude of Ca^{2+} flux (and its return to background level) increases with the duration of light exposure, showing that the amount of calcium that may be mobilized is accumulated in chloroplasts during daytime. If

the lights are turned on before calcium elevation, no increase in free Ca^{2+} is obtained in the stroma. The Ca^{2+} spike is attenuated also if lights are turned on during the increasing phase of Ca^{2+} elevation. The expected biological consequence of these large Ca^{2+} increases in the stroma is the inhibition of photosynthetic processes. The intriguing fact is that inhibiting photosynthetic electron transport has only a poor effect on the magnitude of the Ca^{2+} increases in the dark. Therefore, the replenishment of the mobilizable Ca^{2+} pool is not strictly dependent upon photosynthesis. Moreover, increases in stromal Ca^{2+} do not correlate with corresponding decreases in cytosolic calcium levels showing that Ca^{2+} flux at lights off comes from the chloroplasts. Darkness induces also Ca^{2+} increases in the cytosol correlatively to the decrease in the chloroplastidic stroma. However, the magnitude of the cytosolic burst is 3 to 4-fold lower suggesting that most of the Ca^{2+} is remobilized by chloroplasts. Collectively, these dark-dependent changes in Ca^{2+} in both the cytosol and the chloroplast may signal the end of the day to the cell [15].

2.2. Calcium movements and biological effects in isolated and cellular plant mitochondria

Mitochondria isolated from different plants are able to take up calcium (at concentrations as low as a few μM) from the medium in the presence of respiratory substrates [16].

Data obtained with oat mitochondria suggest further that the plant photoreceptor, phytochrome, may regulate Ca^{2+} fluxes in a photoreversible manner [17]. Red light irradiation reduces the net Ca^{2+} uptake which is restored back to the dark control level upon far-red irradiation. In the presence of ruthenium red (an inhibitor of active Ca^{2+} uptake) in the reaction medium, red light irradiation provokes a Ca^{2+} release from the mitochondria *via* a passive efflux mechanism, suggesting that mitochondria are able to take up from and to release Ca^{2+} into the cytosol.

Recent data show that exposure of plant isolated mitochondria to Pi and mM concentrations of Ca^{2+} induces a fast shrinkage followed by a high amplitude swelling [18,19]. Both Pi and Ca^{2+} are required and replacing Ca^{2+} by an equivalent concentration of Mg^{2+} promotes shrinkage but not swelling, showing that the process is ion-specific. Swelling of mitochondria reflects an expansion of the matrix that ends up with the rupture of the external membrane and the release of proteins and especially of cytochrome *c*. Further work on potato mitochondria has established that cyclosporin A inhibits Ca^{2}-induced swelling even after collapsing the $\Delta\psi$. Collectively, these data suggest the occurrence of permeability transition pores (PTP) located at the contact between inner and outer membranes in plant mitochondria. This process referred to as mitochondrial permeability transition is known to allow mitochondria to be cellular stress sensors and central players in cell death in animals [20]. Mitochondrial Ca^{2+} overload activates PTP resulting in transient mitochondrial depolarization and decreased ATP production. PTP gating may also cause release of mitochondrial proteins (and especially cytochrome *c*) that activate apoptotic pathways. Interestingly, under anoxia (leading to accelerated ATP depletion and Pi increased levels), the onset of the Ca^{2+}-induced swelling as well as the rate of the process are accelerated and indicate that, in vitro, plant mitochondria undergo a faster mitochondrial permeability transition [18,19]. It has been shown that in response to oxidative stress, increases in electron transport in mitochondria trigger H_2O_2 production, depletion of ATP, and opening of PTP and cell death [21].

2.3. Calcium movements and biological effects in isolated and cellular nuclei

Early experiments have shown that nuclei isolated from plants are able to phosphorylate proteins in a calcium dependent manner [22]. Data accumulated over years have refined these initial results by characterizing a number of Ca^{2+}-binding or regulated proteins in the nucleus. These include calmodulin, annexin, transcription factors and calcium-dependent protein kinases and phosphatases [23,24]. Recently, it has been shown that a Ca^{2+}-calmodulin-dependent kinase necessary for the establishment of the symbiotic association between nitrogen fixing bacteria and plant legumes is located in the nucleus [25]. The nucleus is separated from other cell compartments by a double membrane punctuated by nuclear pore complexes (NPC). NPCs allow trafficking of molecules and ions between the cytosol and the nucleoplasm. Calcium permeation through NPC by simple diffusion fully explains the pattern of nuclear calcium upon stimulation of cardiac myocyte [26]. However, different lines of research performed mainly on animal systems have shown that calcium channels and transporters localize to the nuclear envelope [27–29]. These data suggest that the nucleus is equipped to generate calcium signals, and the diffusion hypothesis may not explain all nuclear calcium patterns.

In plants, nuclei isolated from tobacco cells, harboring aequorin in the nucleoplasm, respond to chemicals like mastoparan [30], to temperature changes or to mechanical stimulation [31] by large $\Delta[Ca^{2+}]_{nuc}$. Incubation of nuclei in a medium containing high concentrations of Ca^{2+} has no effect on nucleoplasmic calcium in the absence of stimulation, ruling out the possibility of a passive diffusion from the incubation medium. Symmetrically, chelating extra nuclear calcium with EGTA does not inhibit the increase in free nucleoplasmic Ca^{2+} elicited by mechanical or thermal stimuli, establishing that the signal Ca^{2+} is mobilized from the nucleus itself [31]. Because of its ability to accumulate calcium, in the lumen, the nuclear envelope may be the source of the mobilized calcium.

Based on the above-mentioned data, calcium homeostasis and disturbance in plant nucleoplasm can be described and simulated by a simple mathematical model [32]. The model considers the isolated nucleus as a closed system composed of two compartments: the nucleoplasm (where $\Delta[Ca^{2+}]_{nuc}$ takes place) and a calcium store (corresponding to the nuclear envelope). Calcium channels located to the inner nuclear membrane mobilize the accumulated calcium which is then released into the nucleoplasm. An elusive calcium transporter located to the inner membrane is predicted to expel calcium from the nucleoplasm and replenish the lumen [32].

Patch-clamping nuclear membrane reveals the existence of non-selective voltage-dependent Ca^{2+}-channels in beet [33]. Moreover, Ca^{2+}-ATPases pumps localize to the outer membrane of the nuclear envelope isolated from tobacco cells [34]. In animals, the inner membrane of nuclear envelope contains different ligand operated channels [35]. Patch-clamping of nuclei isolated from osteoblastic-like MC3T3-E1 cells allows characterizing mechano-sensitive Ca^{2+}-channels responsible for calcium elevation [36]. In animals, TRP-like channels are known to sense either temperature or pH [37,38] but their presence in the nuclear compartment has not been established. In nuclei isolated from tobacco cells, $\Delta[Ca^{2+}]_{nuc}$ elicited by either pH and or temperature changes are inhibited by drugs known to inhibit TRP-like channels [31].

2.4. The dynamics of cytosolic and organelle calcium are differentially regulated

The data described in the preceding paragraphs are indicative of the ability of chloroplasts, mitochondria and nuclei to generate intra organelle changes in free Ca^{2+} concentrations. The availability of bioluminescent or fluorescent Ca^{2+}-probes that may be targeted to a particular compartment allows determining relationships between the organelles [39].

Thus, pulses of blue light induce $\Delta[Ca^{2+}]_{cyt}$ in *Arabidopsis* and tobacco seedlings [40]. The spectral response of the Ca^{2+} transient is similar to phototropism and suggests the involvement of NPH1 as the photoreceptor. Use of organelle-targeted aequorin either in the nucleus or the chloroplasts shows that Ca^{2+} increases occur only in the cytosol. These observations suggest that physiological responses implicating NPH1 may be specifically transduced through $[Ca^{2+}]_{cyt}$. A more complex situation has been described depending upon the physiological conditions experienced by plants.

Thus, under continuous illumination of tobacco plantlet free Ca^{2+} concentrations vary rhythmically in the cytosol [41]; upon transfer to darkness the periodic fluctuations stop and resume upon turning lights on. The oscillations may be phase-shifted by dark–light transitions and present the characteristics of a circadian rhythm. Interestingly, in contrast to the cytosolic compartment, there is no circadian variation in $\Delta[Ca^{2+}]_{chl}$ under continuous illumination. Rather, on transfer to darkness, a large increase followed by circadian oscillations in $\Delta[Ca^{2+}]_{chl}$ is observed. Therefore, Ca^{2+} oscillations are regulated differentially in the two considered compartments.

Cooperation between mitochondria and the cytosol is clearly shown when plants experience anoxia. In these conditions, mitochondrial Ca^{2+} varies rapidly and reversibly in response to changes in O_2 availability [42]. The $\Delta[Ca^{2+}]_m$ is inversely related to $\Delta[Ca^{2+}]_{cyt}$. Moreover, $\Delta[Ca^{2+}]_{cyt}$ colocalize essentially with mitochondria showing that mitochondria contribute most probably to $\Delta[Ca^{2+}]_{cyt}$ under anoxia and also to its restoration upon returning back to normoxia. Interestingly, when challenged with different stress conditions, tobacco plantlets respond by a rapid and large variation (up to 1 μM) in mitochondrial free Ca^{2+}. Interestingly, $\Delta[Ca^{2+}]_m$ depends upon the nature of the stress and generates differential responses [43]. Thus, coincident variations in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_m$ are induced by cold or osmotic shock with similar temporal kinetics. $\Delta[Ca^{2+}]_m$ is twice lower than $\Delta[Ca^{2+}]_{cyt}$ suggesting that mitochondria may simply buffer $\Delta[Ca^{2+}]_{cyt}$ through uptake. Touch stimulation induces an immediate elevation of $[Ca^{2+}]_{cyt}$ followed by a return to the background level within less than 30 s. Under the same conditions, after an elevation as rapid as in the cytosol, $\Delta[Ca^{2+}]_m$ is maintained at 37% of its measured maximum for at least 1 min. Lastly, challenging plants with hydrogen peroxide induces $\Delta[Ca^{2+}]_m$ faster and longer than $\Delta[Ca^{2+}]_{cyt}$. Collectively, these data show that mitochondria have the potential machinery to discriminate and generate specific Ca^{2+} signaling pathways in response to an array of stimuli.

Studying the biological effect of two distinct stress conditions acting both of $\Delta[Ca^{2+}]_{nuc}$ and $\Delta[Ca^{2+}]_{cyt}$ sheds light on the subtlety of cellular Ca^{2+} signaling. Thus, wind signals induce $\Delta[Ca^{2+}]_{cyt}$ and $\Delta[Ca^{2+}]_{nuc}$ to peak at 0.3 s and 0.6 s, respectively in tobacco seedlings [44]. In response to cold shock, $\Delta[Ca^{2+}]_{cyt}$ peaks at about 4 s whereas the nucleus reacts maximally after 7 s. Consequently, wind stimuli and cold shock implicate distinct calcium signaling pathways. Interestingly both wind and cold shock induce a particular isoform of calmodulin gene referred to as *NpCaM-1* (for *Nicotiana plumbaginifolia Calmodulin gene 1)*. Comparison of Ca^{2+} dynamics with *NpCaM-1* expression after stimulation suggests that $\Delta[Ca^{2+}]_{nuc}$ are the preferential transducers of wind stimulation and $\Delta[Ca^{2+}]_{cyt}$ of cold shock [44].

In line with these data, other abiotic and biotic stimuli elicit specific cytosolic and nuclear calcium patterns. Thus, in response to osmotic constraints of identical intensity (150 mosM) but sensed by the cell as either “tension” or “pressure”, distinct calcium responses are recorded [45]. Hypo-osmotic constraints elicit large $\Delta[Ca^{2+}]_{cyt}$ and $\Delta[Ca^{2+}]_{nuc}$. In contrast, hyper-osmotic constraints with the same intensity induce only a cytosolic response. Moreover, the cell suspensions responded with characteristic nuclear and cytosolic $\Delta[Ca^{2+}]$ patterns as a function of the nature (non-ionic/ionic osmoticum) and the intensity (osmolarity) of the stimulus.

Cryptogein, a polypeptide secreted by the oomycete *Phytophthora infestans*, triggers defense reaction to pathogen attack in tobacco [46]. Cryptogein induces calcium transients in both the cytosol and the nucleus of tobacco cell suspension cultures [47,48]. Interestingly, $\Delta[Ca^{2+}]_{nuc}$ is maximal 15 min after the cytosolic peak and other elicitors provoking equivalent Ca^{2+} changes in the cytosol have no effect on $\Delta[Ca^{2+}]_{nuc}$. Theoretical considerations lead to the conclusion that if calcium signal kinetics in the cytosol and the nucleoplasm differ each other by at least 1 s, then a simple diffusion of calcium from the cytosol to the nucleus is ruled out [49]. In animals cells different regulation of cytosolic and nuclear calcium has been reported [50,51]. As stated above, isolated plant nuclei are able to convert physical constraints into $\Delta[Ca^{2+}]_{nuc}$ [31]. Conversely, when challenged with cryptogein, isolated nuclei do not respond by $\Delta[Ca^{2+}]_{nuc}$. $\Delta[Ca^{2+}]_{nuc}$ triggered by cryptogein are only detectable in intact cells and needs the initial recognition of the elicitor by its receptors localizing to the

plasma membrane [48]. Therefore only stimuli whose putative sensors are located onto the nucleus may be converted into $\Delta[Ca^{2+}]_{nuc}$.

These two chosen examples clearly show that $\Delta[Ca^{2+}]_{nuc}$ may be directly generated in response to stimuli but may be also a relatively late event that needs the activation of signaling steps located in the cytosol or/and the integrity of a functional continuum between the plasma membrane and the nucleus.

3. Discussion and prospects

Plant cell organelles surrounded by a double membrane (e.g. mitochondria, chloroplasts and nuclei) have multiple functions in Ca^{2+} signaling, regardless of the needs for high Ca^{2+} level for maintaining membrane structure and intactness. Firstly, they contribute to the regulation of $\Delta[Ca^{2+}]_{cyt}$ by taking up Ca^{2+} from the cytosol and storing the cation as a mobilizable form that may be released again in the cytosol. Secondly, they harbor calcium-dependent processes needing the fine-tuning of free Ca^{2+} in the considered organelle. Thirdly, in each organelle subtle compartmentation of mobilizable and free Ca^{2+} exists most probably (e.g. in chloroplasts, free Ca^{2+} concentrations have to be regulated in the stroma and the thylakoid lumen). Ca^{2+} release in discrete and highly localized region of the nucleus may also be generated by the existence of a nucleoplasmic reticulum identified in animal epithelial cell [50]. This branching intranuclear network forming a continuum with the nuclear envelope and the endoplasmic reticulum functions as a store from which Ca^{2+} may be mobilized at precise location in the nucleus. Although its biological role has not been addressed, comparable structure has been described in plant cell nuclei [52].

All these experimental evidence show that each organelle has the ability to control its own $\Delta[Ca^{2+}]$ and, in this way, is autonomous with respect to the cytosol and is not a simple passive sensor of local $[Ca^{2+}]_{cyt}$ (once the calcium pool is accumulated in the organelle). However, cross-talk between different cellular organelles is crucial in Ca^{2+} signaling in relation to the dynamic localization of Ca^{2+} effectors, the Ca^{2+}-dependent translocation and/or post-translational modifications of proteins and the dynamic intracellular reorganization in response to stimuli.

Thus, a particular calmodulin referred to as CaM53 is post-translationally isoprenylated at its C-terminus domain in Petunia [53]. Blockade of the isoprenoid biosynthesis results in the localization of the protein in the nucleus, showing that isoprenylation may drive the subcellular localization of CaM53. In leaves exposed to light for several days, CaM53 localizes to the plasma membrane whereas the protein accumulates in the nuclear compartment in samples maintained in the dark during the same period. Interestingly, dark exposure on a medium supplemented with sucrose prevents nuclear translocation. Recombinant CaM53 activates glutamate decarboxylase a plant calmodulin dependent enzyme and CaM53 gene rescues yeast cmd1Δ mutant defective in the single gene for CaM by complementation [53]. Therefore, the unusual CaM isoform is functionally active.

Another example of intracellular movements of protein related to calcium signaling is illustrated by the case of OsERG1 protein, a small protein containing a Ca^{2+}-dependent membrane-binding motif (C2- domain) [54]. OsERG1 protein which is mainly located in the cytosol accumulates at the plasma membrane upon challenging rice cells with either a fungal elicitor from *Magnaportha grisea* or a calcium ionophore [54].

Collectively, these data show that a particular calcium-binding protein (effector) may localize to a particular compartment as a function of the physiological status of the cell (metabolic status, defense responses). Such versatility may have a profound influence on the coordination of calcium-dependent events in the plasma membrane and the nucleus (CaM53) or in the cytosol and the plasma membrane (C2-proteins) using identical effector protein.

Ca^{2+} by itself may be involved in protein translocation/import into organelles and secretion.

For example, chloroplast imports of a subset of proteins that need a cleavable transit peptide like the small subunit of Ribulose 1, 5-Bisphosphate carboxylase/oxygenase are inhibited either by calmodulin inhibitors or calcium ionophore. Addition of external calmodulin or calcium restores the import process [55]. In growing pollen tube, Ca^{2+}-dependent protein kinase activity is highly concentrated in the apical region [56]. According to Moutinho et al. [56] the modification of growth direction or localized $\Delta[Ca^{2+}]_{cyt}$ leading to reorientation greatly increased the kinase activity presumably in connection with Ca^{2+} -mediated exocytosis.

Local changes in $\Delta[Ca^{2+}]$ in a given compartment may impact on the protein (and other metabolites) repertoire of the others. In the end, the dynamics of the exchanges that allow redistribution of key components of the signaling pathway (enzymes, substrates, transcription factors) determines the biological output and the mounting of an adaptive response.

Not only macromolecules are redistributed but the intracellular architecture may be reorganized also, in response to stimuli. Due to the large number of examples, we will just quote: migration of the nucleus at the site of application of a pathogen most probably driven by cytoskeleton reorganization, local contacts between the endoplasmic reticulum and mitochondria or nuclei. Because the diffusion rate of Ca^{2+} is very slow, the formation of clusters of organelles facilitates contacts between Ca^{2+} stores, calcium channels and transporters and reduces the transit time of information, and increases the efficiency of the overall system.

An exciting challenge in plant Ca^{2+} signaling is an integrative approach allowing quantifying and putting in a logical order different steps that link an initial stimulus to time and space changes in calcium and ending with a biological quantifiable output. Importantly, time-lapse changes in local Ca^{2+} and other second messengers (the proton, active oxygen species, and lipid-derived compounds) should be carefully established. Opportune models to cope with such difficult tasks are already available in plant biology and include physiology of stomata, cell growth (pollen tube) and symbiotic association between nitrogen-fixing bacteria and legumes [5]. Because genomic as well as genetic and cell biology resources are

becoming available, other systems might be of value to consider less specialized but specific aspects of plant physiology [46,57]. We believe that an integrated and multifaceted approach aiming at understanding how plant specific metabolisms are reoriented in response to quantifiable stimuli is worth studying. The topic should give clues on the coupling between Ca^{2+} signaling, gene expression, metabolite production/use and transport in space and time, and how the metabolite phenotype might be controlled by acting on signaling processes.

Acknowledgements

Due to space limitation, we apologize for not having quoted many contributions to the field of calcium signaling in plants.

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