Signaling and Communication in Plants

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Editor

Coding and Decoding of Calcium Signals in Plants

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Preface

Plants cannot move away from their environments. As a result, all plants that survive to date have evolved sophisticated signaling mechanisms that allow them to perceive, respond, and adapt to the constantly changing environmental conditions. Among the many cellular processes that respond to environmental changes, elevation of calcium levels is by far the most universal messenger that couple the primary signals to the cellular responses. It has been puzzling how calcium, a simple cation, translates so many different signals into distinct responses – how is the "specificity" of signal–response coupling encoded within the calcium changes?

Recent research has established a concept called the "calcium signature": each different signal produces a unique calcium change. Such changes entail not only an elevation in concentration but also changes in the temporal and spatial patterns. In other words, a primary signal activates a number of calcium channels and/or pumps located in the various compartments of a plant cell resulting in fluxes of calcium in a particular space with a unique time course. For instance, a signal can produce a calcium "wave" (or a spiking pattern) along the time course in a particular compartment such as cytosol or nucleus. The combination of these temporal and spatial parameters constitutes a four-dimensional pattern unique to an external signal and thus forms the "signature" of each signal. To the plant cell, each calcium signature serves as a secret "code" with specific meaning for cellular response. The molecular components that mediate and regulate the calcium fluxes are involved in the "coding" processes of calcium signals. In order to translate a code into the changes in cellular activities, a cell must be equipped with mechanisms that interpret the meaning of a specific code through the "decoding" process. The molecules involved in the decoding can be referred to as decoders. Therefore, all calcium signaling pathways in plants (or animals) consist of coding and decoding processes, and research in this field is this all about understanding these coding and decoding mechanisms. I thus find the name of this book broadly covering activities in plant calcium signaling research.

Starting with a historical perspective from a pioneer of plant calcium signaling, this book introduces the recent advances in our knowledge of calcium signaling in

various model systems including stomatal guard cells, pollen tubes, and root hairs; followed by coverage of calcium channels in both plants and algal systems exploring evolutionary relationship of the "coding" process; and finished with a variety of molecular players in the "decoding" processes. In all chapters, readers will find the basic background information, current state-of-art in the subject matter, and emerging topics or perspective on the challenges ahead. Indeed, this book is a condensed volume that will provide students as well as advanced researchers a handy and informative resource for a comprehensive understanding of this exciting area of research in plant signal transduction.

Berkeley CA, USA Sheng Luan
February 2011

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Plant Cell Calcium, Past and Future

Anthony J. Trewavas

Abstract Research on two animal cells, *Aequorea victoria* and the Medaka egg, has been instrumental in outlining critical elements to Calcium [Ca^{2+}] signalling in plant cells. Ca^{2+} is coupled to a complex, densely crosslinked network of kinases and phosphatases. It is indicated that future research might concentrate on phase transitions in the cytoplasmic gel structure. In addition, investigations into the complex language structure that underpins the Ca^{2+}-dependent kinase/phosphatase network should advance understanding enormously.

1 Calcium Past

Calcium, [Ca^{2+}], is a ubiquitous ion found in all living systems. The functions of Ca^{2+} in the formation of shells and bones are well known, but receive no further consideration in this book that concentrates instead on the involvement of Ca^{2+} in signalling. The basic elements of Ca^{2+} signalling, in both plants and animals, have proved remarkably similar. Research on two animal organisms has outlined the basic transduction mechanisms. So no apology is offered here for introducing these two animal cells in a book devoted to plant Ca^{2+} research. Each organism has information on different elements of Ca^{2+} signalling. A brief description of them serves to explain some of the subject matter considered later by other authors.

Aequorea Victoria is a small, medusoid jelly fish that emits an intense flash of luminescent light when prodded or attacked by small fish. Aequorin, the soluble protein (plus cofactor) responsible for luminescent light emission, was isolated with some difficulty nearly 50 years ago. Once in the test tube it was early established

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that aequorin emitted luminescent light when treated with Ca^{2+} concentrations substantially less than 1 μM. Since seawater contains approximately 10 mM Ca^{2+}, several obvious conclusions could be drawn. (1) Cytoplasm must be maintained at concentrations of Ca^{2+} well below 1 μM. (2) The external Ca^{2+} in seawater must either be actively excluded or, if any of it enters the cell, be actively extruded. (3) An alarm signal (predation and thus mechanically based) causes a transient elevation of cytoplasmic or cytosolic Ca^{2+}, $[Ca^{2+}]_{cyt}$, which initiates a light flash response. The flashes of light that result from internal transduction of the predatory signal here supposedly attract larger predators of the offending small fish.

Critical observations on the egg of a small ubiquitous fish, the Medaka, added in further important information, this time on the critical spatial dimension to $[Ca^{2+}]_{cyt}$ signalling. When radioactive Ca^{2+} first became available, an early experiment injected both radioactive Ca^{2+} and K^+ in to the squid axon. It was found that whereas K^+ readily diffused, Ca^{2+} stubbornly did not and remained instead at the injection site. The diffusion rate of Ca^{2+} in cytoplasm is now known to be at least 100-fold lower than that in free solution. The Medaka egg is visible to the naked eye and has a micropyle where the sperm enters. By injecting eggs with aequorin, Gilkey et al. (1978) were able to image luminescence, and thus $[Ca^{2+}]_{cyt}$ elevations, after sperm entry. Figure 1 shows the events diagrammatically.

A spatially discrete band (a wave) of higher $[Ca^{2+}]_{cyt}$ estimated at 20 μM is initiated at the sperm entry point and traverses the whole egg, taking about a minute in total. The egg as shown here is seen sideways on; turn the egg through 90° and the wave is seen to be hollow and to be limited to the region directly under the plasma membrane. As the $[Ca^{2+}]_{cyt}$ wave moves, it causes the fusion of cytoplasmic vesicles containing mucilage with the plasma membrane purportedly to prevent polyspermy. A refractory period must exist after the $[Ca^{2+}]_{cyt}$ elevation and decline; otherwise, onward movement of the wave would not occur. Negative feedback may operate to inhibit further $[Ca^{2+}]_{cyt}$ elevation. Ca^{2+} ATPases located in the plasma

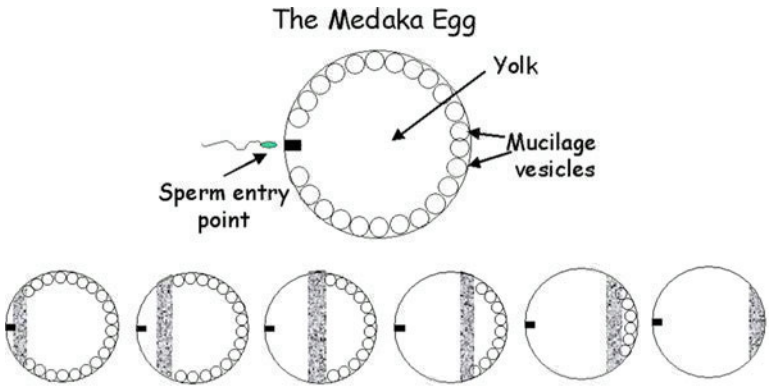

Fig. 1 Selected images of aequorin luminescence (*shaded*) indicating the progress (*from left to right*) of the $[Ca^{2+}]_{cyt}$ wave after fertilisation. Total time = 1 min. Adapted from Gilkey et al. (1978)

membrane and other organelle membranes are activated by elevations of $[Ca^{2+}]_{cyt}$; they commence pumping the excess Ca^{2+} back out of the cytoplasm. Ca^{2+} channels can be closed by protein kinases that are in turn Ca^{2+} activated. If signalling continues then delays in the feedback process can give rise to more permanent oscillations as observed in pollen tubes, root hairs and guard cells. The frequency of oscillation may also be sensed and used as digital information initiating cellular responses.

In the Medaka egg, the initiation and passage of the $[Ca^{2+}]_{cyt}$ wave are essential to initiate embryogenesis. Waves can be initiated in different regions of the egg by calcium ionophores, chemicals that open temporary channels, but embryogenetic initiation then fails. It is, thus, both the spatial and kinetic aspects of the $[Ca^{2+}]_{cyt}$ signal that determines the particular cellular responses. This realisation led to the construction of a whole new technology, that of $[Ca^{2+}]_{cyt}$ imaging, a technology that records both the spatial appearance of elevated $[Ca^{2+}]_{cyt}$ in responsive cells and its kinetics. All cells have a highly structured element to their cytoplasm. Particular protein complexes controlling selected cellular functions are concentrated in certain cytoplasmic regions. $[Ca^{2+}]_{cyt}$ is a signal that provides the potential for the cell to switch on discrete, spatially differentiated cellular responses as required. Chapters that contain information on the pollen tube, root hair and *Fucus* zygote indicate the relevance of imaging technology to understanding plant cell behaviour.

Use of $[Ca^{2+}]_{cyt}$ imaging or plants transformed with aequorin has established that all the major physical, chemical and biological signals that plants experience induce transients or oscillations under experimental conditions. In addition, $[Ca^{2+}]_{cyt}$ is inextricably linked with important developmental phenomena such as polarity after fertilisation or in reproduction or in circadian processes. The main downstream cytoplasmic mechanisms that transduce $[Ca^{2+}]_{cyt}$ transients either use specific intermediary Ca^{2+}-binding proteins such as calmodulin or a heterogeneous group of Ca^{2+}-dependent protein kinases/phosphatases. The advance of molecular technology has provided the vanguard of understanding here, but has also opened a Pandora's Box of complexity in both numbers of families and family members of Ca^{2+}-binding proteins and protein kinases. This book is, therefore, timely in summarising our present state of knowledge. But dealing with the complexity is going to require creative insights and the advance of technology. The information provided in the chapters here should place the challenge directly at the feet of those most able to creatively pick it up.

2 Future Directions for $[Ca^{2+}]_{cyt}$ Research in Plant Cells

Among a number of possibilities I suggest only two. Pollack (2001) in a challenging text places enormous emphasis on the actual physical state of the cytoplasm and the process of phase transition. The cytoplasm is often crudely divided into either gel or sol; the latter is familiar in large plant cells from cytoplasmic streaming. But the business end of the cytoplasm is usually in some form of gel. Gels are familiar objects outside the cell both for electrophoresis and indeed even for consumption.

These gels maintain their shape despite being composed of 95–99% water, the implication being that in the gel, the water must be in some form of structural, even perhaps 'semi-crystalline', arrangement. Cytoplasm, on the other hand, contains anywhere from 20 to 40% protein and it is these macromolecules that are mainly responsible for its gel-like character. But the same requirements for the presence of structured water inside the cytoplasmic gel still hold and Pollack (2001) indicates that structured water may interfere with the ability of proteins to interact with necessary signalling partners. Getting proteins to easily interact with each other would then require a breakdown or removal of the structured water that surrounds them.

It can be anticipated that the cytoplasm will be physically heterogeneous with local areas containing intermediate states from a highly condensed gel to one with much more fluidity. Pollack identifies increased $[Ca^{2+}]_{cyt}$ as a primary agent catalysing phase transition towards a highly condensed gel that squeezes much structured water out, thus increasing protein–protein interaction such as, for example, between kinase and substrate. It should be possible to design appropriate dyes to image where in the cytoplasm these events happen and to correlate them if possible with the distribution of particular cytoplasmic proteins, particularly protein kinases and their substrates.

It is the gel-like cytoplasm that adheres to the plasma membrane in single plant cells is crucial in morphogenesis. But it is also how that gel interacts with $[Ca^{2+}]_{cyt}$ that initiates changes in form (Goodwin 1977; Goodwin and Patermichelakis 1979; Goodwin et al. 1983).

I find Pollack's (2001) emphasis on phase transition between kinds of gel structure attractive because a summary of the physical and chemical changes that initiate callus regeneration, break seed and bud dormancy, and promote root formation or abscission almost exactly matches the list of physical and chemical conditions that modify gel formation (Trewavas 1992; Pollack 2001, p. 115). Pollen tubes and root hairs both express oscillations in growth rates and $[Ca^{2+}]_{cyt}$ with the peak of the former leading the peak of the latter. This is entirely explicable based on the notion that increased $[Ca^{2+}]_{cyt}$ would cause gel contraction and temporarily diminish growth rate.

The alternative direction for research I am suggesting here is presently a more popular route in systems biology. Molecular studies have indicated that cellular proteins from a number of organisms form complex interacting networks; Blow (2009) provides examples of a number of them. Molecular networks can be seen as analogous in some aspects of behaviour to simple neural networks; both can be examined using the conceptual frameworks of information and information processing (Nurse 2008). The question that Nurse addresses is how cells gather, process, store and use the information they acquire from outside. These, of course, are the remits of signal transduction investigations and thus directly relevant to the chapters in this book.

Biological information can be equated to meaningful communication. The quality of information transferred is determined by the constraints that surround

its sensing and transmission (Trewavas 2009). For example, plant cells gain more information from having separate sensors for red and blue light than they would acquire if they just had one light-sensing pigment only. Informational noise, and there is plenty of it in biological systems, can also interfere with the accuracy of transmission.

The protein network structure can be simply (and simplistically) divided into hubs and connectors, the hubs being proteins that interact with many other proteins and the connectors being proteins that interact with a few. Some plant calmodulins and calcium-activated protein kinases must clearly be hubs and it should be possible to identify which out of the numerous calmodulin and kinase possibilities they actually are. The network structures around these hubs could also be envisaged as dependent on the physical state of the cytoplasm relating to the concepts above concerning structured water.

However, analysis of the network of interactions that surrounds any hub can provide structures that can be described as logic modules. These were discussed by Bray (1995, 2009) in an important consideration of proteins as cellular computational elements. As he clearly indicated, particular simple protein complexes involved in signalling can be seen to act biologically in a similar fashion to the familiar Boolean logic gates of NOR, OR, AND, etc. Some of these 'gates' can be grouped together to form logic modules with particular properties. By this means, a start can be made on constructing a cellular computational structure. Protein kinases in particular are typical elements whose behaviour lends themselves to this kind of analysis.

The aim here as indicated by Nurse (2008) is to try and grasp the complex language structure that cells use to underpin such disparate processes as temporal and spatial order, maintenance of cell integrity, homeostasis, inter- and intra-cell signalling and crucially cell memory among others. Neural networks learn by breaking old connections and opening new ones; protein kinases perform that particular function in metabolic networks. The suspicion is that the language structure of information transfer in cells may be much more similar between organisms than the genetic base might imply. Interpreting that language may then provide the necessary breakthroughs in areas that otherwise could remain recalcitrant.

Nurse (2008) suggests working initially from simple logic modules such as the very common negative feedback. There are plenty of such examples in $[Ca^{2+}]_{cyt}$ signalling as indicated above. By identifying the logic gates these represent and adding in other downstream processes, a more complex logic module can be constructed. It should be possible to portray these computationally and examine their properties and start to construct cellular logic circuits. How information actually flows through the system and the constraints that operate upon it can then emerge. As Nurse (2008) indicates, some better understanding of cellular memory and in due course cellular learning become possible. This area is a new challenge for plant cell studies, but the possibilities for discovery are immense.

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Calcium Signaling and Homeostasis in Nuclei

Christian Mazars, Patrice Thuleau, Valérie Cotelle, and Christian Brière

Abstract Calcium variations occurring in the nucleus and in other calcium-active compartments of the plant cell are contributing to encode information of specificity used by the cell to mount an appropriate response to environmental cues. This chapter deals with calcium signaling in the nucleus and reports on the current knowledge on calcium signals monitored in plant cell nuclei in response to biotic and abiotic stimuli. On the basis of both the experimental and modeling data, evidences of the autonomy of the nucleus which is able to generate its own calcium signals and to maintain its calcium homeostasis by itself are brought. Finally, the biological relevance of such nuclear calcium signals is discussed with regard to the nuclear sub-compartments and the biological activities which are taking place in these sub-compartments.

1 Introduction

Considerable interest and research have been focused on calcium ion (Ca^{2+}) because of its mediating role in signal transduction pathways starting from the perception of the initial stimulus and ending with the final adaptive response. Such interest emerged from the numerous observations that calcium concentration ($[Ca^{2+}]$), mainly cytosolic, varies in response to a multitude of abiotic or biotic stresses as extensively reported by several reviews (Kudla et al. 2010; McAinsh and Pittman 2009; Ng and McAinsh 2003; Sanders et al. 2002; Schroeder et al. 2001; Scrase-Field and Knight 2003; White and Broadley 2003). The fact that these

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increases are heterogeneous but nevertheless specific of the intensity and of the nature of the initial stimulus opened a new avenue of research focused on thorough studies of these calcium responses. These studies led the Hetherington's group to propose the concept of calcium signatures or calcium "fingerprints" which emphasizes the idea that specificity of the final and adaptive response is encrypted by the calcium signal itself. This calcium signal can be defined by parameters of duration, amplitude, frequency and spatial distribution (McAinsh and Hetherington 1998; McAinsh and Pittman 2009). Such concept was revisited and confirmed at the single-cell level in very specialized cells such as the guard cells involved in stomata regulation or the root hair cells involved in the establishment of symbiosis with *rhizobia*. In these cells, the minimal number of Ca^{2+} spiking and the optimum frequency required to achieve the expected response had been clearly defined, although decoding mechanisms still remain unsolved (Allen et al. 2001; Miwa et al. 2006). Current research on calcium signaling is still tackling this crucial question of specificity and how it can be achieved through calcium decoding, in other words how frequency, amplitude and signal localization are deciphered by the numerous Ca^{2+}-dependent effectors encoded by the plant genome (Day et al. 2002). These effectors which add further complexity to the calcium network have the ability to bind to and to be regulated by Ca^{2+} through domains being either the EF-hand motif (Nakayama and Kretsinger 1994) or the C2 domain (Cho and Stahelin 2006). Such calcium sensors are involved in protein–protein interactions necessary to regulate the calcium signal itself or to decode it through downstream signaling platforms. The challenging goal is thus to understand how these signaling networks, resulting from the interplay between calcium-binding proteins and their targets, can direct the signaling pathway toward the right and specific final response. Significant progress in understanding these specificity mechanisms has been made through studies related to Ca^{2+}-dependent *P*rotein *K*inases (CPKs) (Boudsocq et al. 2010; see Harmon Chap. 9) or CBL/CIPK (*C*alcineurin *B*-*L*ike calcium-binding protein/*C*BL-*I*nteracting *P*rotein *K*inase) networks recently reviewed (Batistic and Kudla 2004, 2009; Luan 2009; Luan et al. 2002; Weinl and Kudla 2009; and see Chapter "Decoding of calcium signal through calmodulin: calmodulin-binding proteins in plants"). In order to better understand how specificity is established, another parameter of the calcium signal to be considered is the calcium compartmentation. If it is well admitted that cytosolic calcium signals can be interpreted only in 3D (space, time and amplitude), it appears that the "space" component may have different meanings depending on whether it refers to the organ, tissue, cell, organelle or to a sub-compartment of the organelle. Thus, spatio-temporal calcium changes can take place within compartments different from the cytosol such as mitochondria, chloroplast or nucleus (Johnson et al. 1995; Logan and Knight 2003; Xiong et al. 2006) or in small microdomains mainly associated with elementary Ca^{2+} release events as reviewed in animals (Laude and Simpson 2009). Organelles play a major role in generating, modulating and decoding Ca^{2+} signals that can contribute alone or in combination with their cytosolic counterparts to the specificity of the final adaptive response. In plants, scarce data exist concerning calcium signals in organelles (Johnson et al. 1995; Logan and Knight 2003), and efforts have

been concentrated on the nucleus during these last years (as reviewed in Xiong et al. 2006). The investment on nuclear calcium signaling has been motivated by the functional originality of the nucleus which is able to orchestrate different activities such as transcription regulation (Finkler et al. 2007; Galon et al. 2009; Kim et al. 2009), protein import during retrograde signaling from the plastid to the nucleus and protein export during anterograde signaling from the nucleus to the plastid (Inaba 2010), spatial organization of the genome (Saez-Vasquez and Gadal 2010), as well as by the need to improve the knowledge on calcium-regulated nuclear activities and the mechanisms involved. This chapter attempts to review the state of the art on calcium signaling in the nucleus.

2 Plant Cell Nuclei Are Able to Generate Calcium Signals in Response to Exogenous Stimuli

Nuclear calcium signaling was initially investigated in the nucleus of different animal cell types using fluorescent calcium probes (for review, see Bootman et al. 2000). However, it was rapidly shown that these probes behave differently in the nucleus in terms of affinity for calcium and dynamic range in comparison with the cytosol. As a consequence, the measurements of calcium variations within the nucleus were considered as being not entirely reliable (O'Malley et al. 1999; Thomas et al. 2000) and led to refute the idea that the nucleus was able to produce calcium signals by itself.

The use of protein-based calcium probes, and especially the recombinant aequorin technology, has allowed to overcome these problems (Knight et al. 1991; Nakajima-Shimada et al. 1991). Aequorin is a luminescent protein found in the jellyfish (*Aequorea victoria*) (Shimomura et al. 1962) and is composed of a calcium-binding protein (apoaequorin) and a prosthetic group, the coelenterazine (a luciferin molecule). Upon calcium binding, coelenterazine is spontaneously oxidized and the whole complex emits blue luminescent light proportionally to the concentration of free calcium (Shimomura et al. 1962). The successful cloning of aequorin cDNA (Inouye et al. 1985) permitted the development of recombinant technology allowing organisms to be stably transformed with the apoaequorin gene and to address the protein in the cytosol or in any intra-cellular organelle, with the appropriate addressing sequence (Rizzuto et al. 1993). Thus in plants, using a chimeric protein formed with the aequorin protein fused to nucleoplasmin, the group of Anthony Trewavas has been able in the late 1990s to monitor, for the first time in plant cells, nuclear Ca^{2+} variations in response to abiotic stimuli (van der Luit et al. 1999). Particularly, they showed that challenging intact tobacco (*Nicotiana plumbaginifolia*) seedlings with either wind or cold shock resulted in $[Ca^{2+}]$ changes both in the cytosol and the nucleus. Because nuclear $[Ca^{2+}]$ increases were always delayed with respect to the cytosolic transients, it may be concluded that these two stimuli activate distinct Ca^{2+} signaling pathways (van der Luit et al. 1999).

Using aequorin-transformed tobacco BY-2 cells (Mithofer and Mazars 2002), it was further shown that lowering the osmolarity of the culture medium increased the cytosolic [Ca^{2+}] in a bimodal manner while a rapid mono-phasic increase in nuclear [Ca^{2+}] concomitant with the first cytosolic Ca^{2+} peak was observed. In contrast, increasing the osmolarity elicited a smaller but identical biphasic response in cytosolic [Ca^{2+}] without inducing changes in nuclear [Ca^{2+}] (Pauly et al. 2001). In the same way, it has been shown that cryptogein, a polypeptide secreted by the oomycete *Phytophthora cryptogea,* which triggers defense reaction to pathogen attack in tobacco (Lecourieux et al. 2006), induced calcium transients both in the cytosol and the nucleus of tobacco cells (Lecourieux et al. 2002, 2005). Interestingly, nuclear Ca^{2+} variations occurred 15 min after the cytosolic Ca^{2+} peak, suggesting that increases of [Ca^{2+}] in the nucleus were likely not due to a simple diffusion of calcium from the cytosol. Altogether these data demonstrated that changes in cellular [Ca^{2+}] may proceed differently in cell compartments and that modification of nuclear [Ca^{2+}] may be disconnected from cytosolic Ca^{2+} transients.

Another example of the involvement of nuclear calcium in plant biology is the finding concerning the initiation of the symbiotic interaction between legumes and *rhizobia*. A key step in this initiation involves the perception by the host roots of specific lipochitooligosaccharides, known as nodulation factors or Nod factors (NFs) (Lerouge et al. 1990). When perceived, NFs activate a number of cellular responses in root cells, including early ion fluxes (especially Ca^{2+}), membrane depolarization, cytoplasmic alkalinization and delayed intracellular Ca^{2+} oscillations, which in turn, lead to expression of specific genes such as the early nodulin genes associated with nodule formation (Oldroyd and Downie 2008). Mutants impaired in NF-induced Ca^{2+} oscillations do not exhibit nodulation, showing that the Ca^{2+} oscillations are essential for the nodulation process (Miwa et al. 2006; Walker et al. 2000). Very recently, using a nuclear-targeted calcium reporter protein (the cameleon protein YC2.1), it has been demonstrated that NFs triggered Ca^{2+} oscillations within the nucleus in the legume *Medicago truncatula* (Sieberer et al. 2009). These Ca^{2+} oscillations would then be sensed by a *C*alcium/*CaM*odulin-dependent protein *K*inase CCaMK (DMI3, for *D*oesn't *M*ake *I*nfections 3), a presumed Ca^{2+} decoder, which has been shown to be exclusively located within the nucleus in *M. truncatula* root hair cells (Smit et al. 2005 and see below).

3 Nuclear Calcium Signals May Be Disconnected from Cytosolic Calcium Signals

An important question focuses on the idea that the nucleus can have an autonomous calcium signaling system and is able to control its own calcium homeostasis by itself. Until recently, it was considered that calcium ions were able to diffuse freely through the numerous pores which punctuate the *N*uclear *E*nvelope (NE), namely the *N*uclear *P*ore *C*omplexes (NPCs), rendering the question of an autonomous

calcium signaling in the nucleus a very controversial issue. Indeed, NPCs in animal nuclei and more specifically in *Xenopus* have an averaged diameter of 110–120 nm (Goldberg and Allen 1996) that should allow free Ca^{2+} diffusion and prevent the formation of nuclear/cytosolic Ca^{2+} gradients. Although few years ago scientific evidences were obtained in animal cells against calcium diffusion through the nuclear pores (al-Mohanna et al. 1994), the fact that authors used fluorescent calcium probes and that the fluorescence output of these Ca^{2+} indicator dyes is altered by their cytoplasmic or nucleoplasmic environment (see above, section "Plant cell nuclei are able to generate calcium signals in response to exogenous stimuli") led people to consider that these results were artifacts. To circumvent these technical problems arising with the Ca^{2+} fluorescent dyes, the Ca^{2+}-sensitive photoprotein aequorin was used (Badminton et al. 1995, 1996), but led also to discrepant results (Brini et al. 1993), thus strengthening the dominant paradigm of free cytosolic Ca^{2+} diffusion through NPCs in animal cells.

In plants, the architecture of the NE is similar, at least in terms of presence of NPCs, to the architecture of the NE described in nuclei of animal cells (Xu and Meier 2008). A recent work carried out on tobacco BY-2 cells, which have been the main cellular model used to study nuclear calcium, indicates that plant NPCs are closely related to vertebrate NPCs. They appear highly organized on the nuclear surface with a number and an arrangement depending upon the proliferating or stationary phases of cells. They are distributed with one of the highest densities measured in eukaryotes (40–50 NPCs per μm^2) and are larger than the yeast NPCs (95 nm) but smaller than those of *Xenopus* (110–120 nm) (Fiserova et al. 2009). From these data it would be expected that observations similar to those made in animal cells should be reported in plants, pointing out nuclear-cytosolic Ca^{2+} gradients in some situations and calcium diffusion through the NPCs in other situations.

As mentioned above (section "Plant cell nuclei are able to generate calcium signals in response to exogenous stimuli"), it has been suggested that in response to both biotic and abiotic situations, nuclear Ca^{2+} signals may not result from the free diffusion of cytosolic Ca^{2+} through the NPCs. The different studies performed on tobacco cells have clearly shown that the delay between the cytosolic Ca^{2+} peak and the nuclear Ca^{2+} peak could range from seconds in response to mastoparan (Pauly et al. 2000) to minutes in response to osmotic shocks (Pauly et al. 2001), elicitors (Lecourieux et al. 2005, 2006) and sphingolipids (Lachaud et al. 2010; Xiong et al. 2008) and up to 1 h in response to some oxylipins (Walter et al. 2007). Such results strongly suggest that nuclear calcium transients are generated from inside the nucleus and not from the cytosol and that nucleus is thus completely autonomous in terms of calcium regulation. This hypothesis was strengthened by the fact that isolated and purified nuclei from tobacco BY-2 cells were able to directly generate Ca^{2+} transients in response to mechanical shocks, temperature variation or chemicals such as mastoparan and sphingolipids (Pauly et al. 2000; Xiong et al. 2004, 2008). In addition, incubation of tobacco nuclei in a medium containing high concentrations of Ca^{2+} had no effect on nucleoplasmic calcium, ruling out the possibility of a passive diffusion from the incubation medium. Conversely, chelating extra-nuclear calcium with EGTA did not inhibit the increase in free

nucleoplasmic [Ca^{2+}] elicited by mechanical or thermal stimuli, establishing that the Ca^{2+} signal was mobilized from the nucleus itself (Xiong et al. 2004). More recently, a structure–function study conducted with jasmonate derivatives has shown that jasmonate isoleucine was able to generate a nuclear Ca^{2+} signal without any measurable cytosolic Ca^{2+} response (Walter et al. 2007). All these results strongly suggest that in plant cells, the nucleus possesses the ability to regulate its Ca^{2+} homeostasis by itself. It is also noteworthy that recent studies from the animal field clearly argue against the concept of calcium diffusion and clearly show that the nucleus autonomy in terms of Ca^{2+} signaling is not restricted to plants (Rodrigues et al. 2009).

Although these different data demonstrate the independence of the nucleus toward the cytosol, it cannot be ruled out that in response to some still unknown stimuli, the nuclear calcium signaling machinery needs to be activated by intermediate effectors located in the cytosol.

4 Machinery Implicated in the Nuclear Calcium Homeostasis and Sensing

4.1 Regulation of Nuclear Calcium Homeostasis

To regulate the calcium homeostasis, the nucleus has to host its own mobilizable sources of Ca^{2+} and the whole associated machinery (channels and transporters) to generate and to pattern the calcium signals. Thus the main nuclear calcium store has long been supposed to be located in the perinuclear space corresponding to the lumen between the outer and inner membranes of the NE. However, this view has recently evolved with the observation of nuclear invaginations of the NE inside the nucleoplasm that can bring calcium sources close to important functional subcompartments. Such structures, called nucleoplasmic reticulum in animals (Echevarria et al. 2003), have also been observed in tobacco cells (Collings et al. 2000, Mazars et al. unpublished), but their role as potential intranuclear calcium sources still waits confirmation in plants. Other potential calcium stores observed in animal nuclei are small vesicular Ca^{2+} stores containing high-capacity Ca^{2+} buffering proteins called chromogranins (Yoo et al. 2005). To our knowledge, such vesicular nuclear calcium stores have not yet been reported in plants.

A minimum set of passive and active effectors necessary for the patterning of the nuclear calcium signal has also to be present on the inner and outer membranes of the NE to allow an independent calcium signaling in the nucleus. The current knowledge on the components of the plant nuclear envelope is still very limited in comparison with what is known in animals, and recent reviews on the plant nucleus envelope (Meier 2007; Meier and Brkljacic 2009) do not mention the existence of additional components of the calcium toolkit (Berridge et al. 2000; Kudla et al. 2010) that could take part in the patterning of calcium signals within the

nucleus. In contrast to animal studies, proteomic analyses of NEs and NPCs have not yet been carried out in plants, *de facto* excluding the characterization of any new putative calcium channel or calcium transporter in plant nuclear membranes. The only available information concerns an immunochemical approach showing the labeling of a putative Ca^{2+}-ATPase on the cytosolic side of the outer membrane of the NE (Downie et al. 1998). To date, the only evidences for calcium channels come from electrophysiological approaches conducted on NPC of nuclei prepared from red beet (Grygorczyk and Grygorczyk 1998). However, data mining of the current available *A. thaliana* databases has allowed the possibility to predict putative transporters or calcium effectors containing bipartite *N*uclear *L*ocalization *S*ignal (NLS)-like sequences (Matzke et al. 2001). Thus, using the INTERPRO domain database, Matzke and collaborators screened various families of recognized and putative ion transport proteins in *Arabidopsis* for potential bipartite NLSs. They found 6 out of 18 P-type ATPases capable of catalyzing cation uptake and/or efflux, 3 out of 19 probable cyclic nucleotide gated channels and 2 out of 15 K^+ channels that could modulate calcium channels as suggested for CASTOR and POLLUX channels in *Lotus japonicus* (Matzke et al. 2009). The formal characterization of these putative nuclear transporters/channels remains to be done, and it may be anticipated that some of them may have been missed because they lack canonical NLS (Lange et al. 2007) or because potential channels whose sequences are not known (i.e., *I*nositol 1,4,5-*t*ris*P*hosphate (IP_3) receptors) could not be considered in the screening process. Nevertheless, the data obtained through the *in silico* approach reinforce the hypothesis that the inner membrane of the NE might contribute to nuclear calcium homeostasis regulation, although the machinery that could explain this nuclear calcium homeostasis still remains to be discovered.

4.2 Decoding of Nuclear Calcium Signals

The nuclear calcium signature can be decoded by calcium sensors which include *CaM*odulin (CaM) and *C*alcium-*D*ependent *P*rotein *K*inase (CDPK or CPK). The presence of CaM in the nucleus and the identification of several nuclear CaM-binding proteins (see Poovaiah Chap. 7) suggest an important role for CaM as a primary calcium decoder in this compartment. Thus, CaM 53, one of the members of the large calmodulin family in plants, has been shown to localize at the plasma membrane or in the nucleus, depending on its prenylation status on a C-terminal domain (Rodriguez-Concepcion et al. 1999). The subcellular localization of prenylated CaM 53 at the plasma membrane can be altered by a block in isoprenoid biosynthesis, by sugar depletion or by dark conditions, leading to a localization of the protein in the nucleus. These results suggest that CaM 53, in concert with calcium signals, could activate different targets in response to metabolic changes. Moreover, the role of CaM in the nucleus is emphasized by the identification of numerous nuclear *CaM-B*inding *P*roteins (CaMBP), as the pea apyrase (Hsieh et al. 2000), the *P*otato *C*aM-*B*inding *P*rotein (PCBP) (Reddy et al. 2002) or AtCaMBP25 that

functions as a negative regulator of osmotic stress responses in *Arabidopsis thaliana* (Perruc et al. 2004). More recently, a gene coding a Ca^{2+}- and CaM-dependent protein kinase (CCaMK) required for bacterial and fungal symbioses has been cloned in *Medicago truncatula* (Levy et al. 2004). This nuclear CCaMK called DMI3 binds to CaM in a Ca^{2+}-dependent manner (Sathyanarayanan et al. 2000) and has been shown to interact with a nuclear protein of unknown function (Messinese et al. 2007).

Another evidence supporting the role of CaM as an important calcium decoder in the nucleus comes from the characterization of a variety of transcription factors directly regulated by Ca^{2+}/CaM, such as WRKYs, MYBs and *CaM*-binding *T*ranscription *A*ctivators (CAMTAs) (for review, see Galon et al. 2009). These CAMTAs possess a novel type of DNA-binding domain, termed CG-1, which contains a predicted bipartite NLS. It has been clearly shown that these transcription factors are targeted to the nucleus where they activate the transcription (Bouché et al. 2002). The CAMTA-binding motifs encompass the *ABA-R*esponsive *cis-E*lements (ABREs) such as the classical ABRE [CACGTG(T/G/C)] and the ABRE-CE *C*oupling *E*lement [(C/A)ACGCG(T/G/C)], two sequences identified as Ca^{2+}-responsive *cis*-elements (Finkler et al. 2007; Kaplan et al. 2006). Other plant transcription factors can directly be regulated by Ca^{2+} or indirectly through Ca^{2+}-dependent phosphorylation/dephosphorylation activities (Galon et al. 2009).

Indeed some *P*rotein *K*inases (PKs) belonging to various families of Ca^{2+}-regulated PKs, i.e. CPK or CDPK (Batistic and Kudla 2009; Harper et al. 2004; Hrabak et al. 2003) or calcineurin B-like interacting protein kinases (Batistic and Kudla 2009), have been found in the nucleus (for review see Dahan et al. 2010). Thus, based on GFP-fused proteins, AtCPK3, AtCPK4, AtCPK11 and AtCPK32 are localized both in the cytosol and the nucleus (Dammann et al. 2003). Similarly, in a work aiming at deciphering the role of CDPKs in plant innate immune signaling, Boudsocq and collaborators confirmed the double localization of AtCPK4 and AtCPK11 but in addition they found similar localizations for AtCPK5 and AtCPK6 (Boudsocq et al. 2010). In a recent study devoted to salt-stress signaling in Arabidopsis, it has been shown using YFP-fusion proteins transiently expressed in leaf epidermal cells that CPK3-YFP predominantly localized at cellular membranes and in the nucleus (Mehlmer et al. 2010).

4.3 *Proposed Mechanisms of Nuclear Calcium Signaling and Homeostasis*

The various data described above have shown that the nucleus likely possesses elements of the calcium machinery necessary to generate and control calcium changes in the nucleoplasm: calcium stores (e.g., the perinuclear space), Ca^{2+} channels, Ca^{2+} transporters and Ca^{2+} buffers (the term "Ca^{2+} buffer" refers here to chemical species acting as calcium ligands with a rapid equilibrium between the free and bound forms of calcium). Furthermore, experimental data on isolated nuclei of

BY-2 cells led to the conclusion that these nuclei constitute a closed system; they are able to respond to mechanical stimulation in a pH-dependent manner and to regulate resting levels of nuclear [Ca^{2+}] without any exchanges of Ca^{2+} with the external medium (Xiong et al. 2004). This raises the question of what minimal equipment and mechanisms are necessary to generate Ca^{2+} signals and to maintain Ca^{2+} homeostasis in the nucleoplasm. To address this question, Brière et al. (2006) have used a mathematical modeling approach to simulate nucleoplasmic calcium dynamics under various conditions (mechanical stimulus, pH or temperature variation).

The model considers that the nucleus is composed of two physical compartments: the nucleoplasm and the perinuclear space of the nuclear envelope. In each compartment, Ca^{2+} is either in a free form or bound to Ca^{2+} buffers. A rapid increase in the free [Ca^{2+}] in the nucleoplasm, following a mechanical stimulus, may easily be explained by the opening of Ca^{2+} channels located on the inner nuclear membrane, inducing a Ca^{2+} influx from the nuclear store. Explaining the slow decreasing phase of the process, which takes up to 3 min to return to the basal Ca^{2+} level, is not so straightforward. Binding of Ca^{2+} to negatively charged compounds would be a way to lower the concentration of free Ca^{2+} back to its basal level in the nucleoplasm. However, the fast kinetics of Ca^{2+} buffering is not consistent with the observed kinetics. Furthermore, if buffering was the only mechanism involved in regulating the nucleoplasmic Ca^{2+} level, successive stimulations should result in a rapid depletion of the nuclear store. In isolated nuclei stimulated by successive mechanical shocks, such depletion was never observed. On the contrary, successive stimulations of isolated nuclei led to a train of sustained Ca^{2+} peaks (Xiong et al. 2004). A reasonable alternative explanation is an existing balance between putative Ca^{2+} channels and Ca^{2+} transporters located on the inner membrane of the nuclear envelope. Nevertheless, existence of nuclear Ca^{2+} buffers was found to be important for explaining the kinetics of Ca^{2+} changes under various conditions, in particular in response to cold shocks. Thus, according to the model, after a nuclear [Ca^{2+}] elevation induced by a transient mechanical stimulus, restoration of the basal nuclear [Ca^{2+}] would result from the balance between Ca^{2+} release from and Ca^{2+} transport to the perinuclear space, acting in concert with the buffering capacity of the nucleoplasm and the nuclear stores. This is illustrated in Fig. 1 where [Ca^{2+}] variations observed in isolated nuclei in response to a mechanical stimulus are simulated. At acidic pH, most of the free Ca^{2+} in the perinuclear space is mobilized by a stimulus before re-uptake via Ca^{2+} transporters. At more alkaline pH, the nuclei did not convert mechanical stimuli into nuclear [Ca^{2+}] variations but became sensitive to temperature; the model proposes that, in this case, both the release of Ca^{2+} from stores and the Ca^{2+} binding capacity of the nucleoplasm are modified. In contrast to mechanical stimuli, an increase in the temperature of the medium containing the nuclei resulted in nuclear [Ca^{2+}] increases. Simulation of temperature effects led to the proposition that this physical parameter has its effect through the activation of putative channels (by changing membrane dynamics) and the modification of the buffering capacity of the nucleoplasm.

The experimental data used to propose the model presented here show clearly that acidic pH values do not change nucleoplasmic [Ca^{2+}], suggesting that

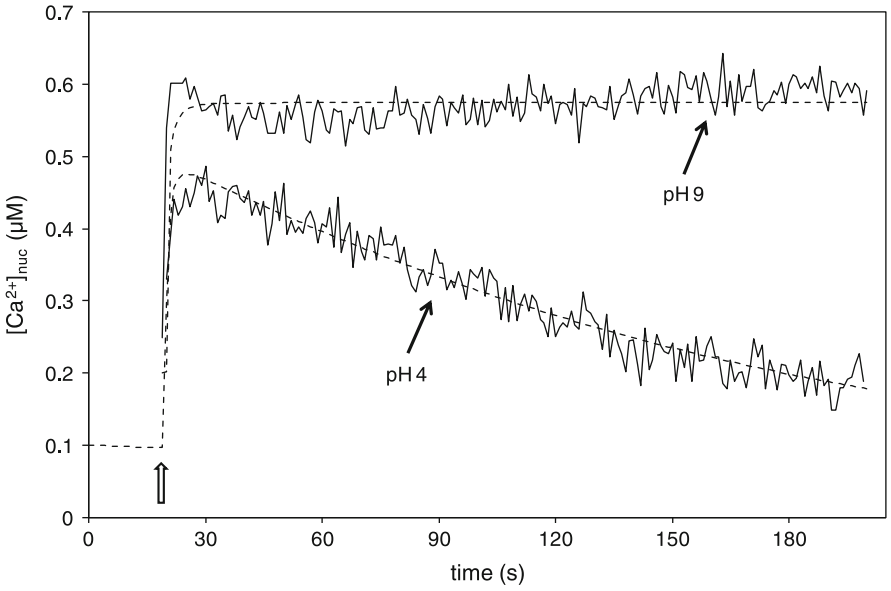

Fig. 1 Calcium variations induced by mechanical stimulation of isolated nuclei at two pH values. *Broken line*: adjustment of the model from Brière et al. (2006) to experimental data (Xiong et al. 2004) (*continuous line*). At acidic pH a mechanical stimulus induces a transient influx of calcium within the nucleoplasm followed by a slow re-uptake to the nuclear store. At basic pH, the nuclei become insensitive to a mechanical shock but a change in temperature modifies the balance between Ca^{2+} influx and Ca^{2+} transport, inducing an elevation of the steady nucleoplasmic Ca^{2+} concentration

*A*cid-*S*ensing *I*on *C*hannels (ASICs)-like channels (Krishtal 2003) are not key players in the process. Moreover, the pharmacological profile of the putative channels is rather more compatible with channels being *T*ransient *R*eceptor *P*otential (TRP)-like or IP_3-dependent channels (Cardenas et al. 2005; Clapham et al. 2001; Malviya 1994). The putative channels become highly sensitive to activation by mechanical stimulations at acidic pH and not at alkaline pH. Changes in ionic charges of the channels may be the mechanism that controls their sensitivity to either mechanical or thermal stimulation. Clearly, the molecular nature of the channels and the mechanism of their activation remain to be clarified.

5 The Biological Relevance of the Autonomy of Nuclear Calcium Signaling

To date, only a few examples can illustrate the biological relevance of the autonomy of nuclear Ca^{2+} signaling. The first one is related to gene expression and comes from the pioneering work of the Trewavas group that indicates that wind-induced

expression of the calmodulin gene *NpCaM-1* in *N. plumbaginifolia* depends predominantly on nuclear calcium signaling (van der Luit et al. 1999).

The second is associated with the bacterial symbiosis process and is related to the activity of a calcium sensor that controls the full process of symbiosis establishment leading to the development of nitrogen-fixing nodules. The DMI3 protein is a CCamK localized in the nucleus harboring a visinin domain (Levy et al. 2004; Mitra et al. 2004) capable of calcium binding (see above). The Ca^{2+} binding to the visinin domain is required for the subsequent association of DMI3 with calmodulin (Sathyanarayanan et al. 2000). Because the removal of the autoinhibitory domain makes the enzyme constitutively active and allows spontaneous nodulation in the absence of stimulation by either NFs or bacteria (Gleason et al. 2006) and because *dmi3* mutants are still able to generate Ca^{2+} oscillations but are defective in developing symbiotic association, one can speculate that nuclear Ca^{2+} signals generated in the nucleus are probably the master regulator of DMI3 protein that influences the subsequent symbiosis events.

The third example is associated with the jasmonate pathway. This pathway has been extensively studied since jasmonates are important regulators of gene transcription during plant growth and in response to biotic or abiotic stresses (Wasternack 2007). The role of calcium in this pathway has been evaluated by measuring the Ca^{2+} responses induced in tobacco BY-2 cells constitutively expressing the aequorin calcium probe. Upon external application of jasmonate derivatives it has been shown that these molecules are able to induce Ca^{2+} variations in both the cytosol and the nucleus or only in the nucleus in the case of jasmonoyl-isoleucine (Walter et al. 2007). Remarkably, this conjugate is the only jasmonate derivative capable of binding the *S*KP1 *C*ullin *F*-box protein E3 ubiquitin ligase (SCF ^{COI1}) and to promote its association with the *JA*smonate *Z*IM-domain (JAZ1) transcriptional repressor leading to its degradation through the 26 S proteasome (Thines et al. 2007). Upon JAZ1 degradation, the transcription factor MYC2 is derepressed, and expression of jasmonate responsive genes is induced (Staswick 2008). Since jasmonoyl-isoleucine is able to generate Ca^{2+} variations only in the nucleus and because it is also associated with derepression of transcription, it is tantalizing to speculate that nuclear calcium specifically controls some steps of this nuclear process.

The last example concerns the apoptosis-like symptoms induced in BY-2 tobacco cells by dihydrosphingosine (DHS), a member of the large family of sphingolipids. The external application of DHS induces Ca^{2+} variations both in the cytosol and in the nucleus of the cells which are followed by *P*rogrammed *C*ell *D*eath (PCD) symptoms (Lachaud et al. 2010). Upon selectively blocking the DHS-induced nuclear Ca^{2+} increases without affecting the cytosolic Ca^{2+} responses, using inhibitors of the *i*onotropic *Glu*tamate *R*eceptors (iGluR), PCD is blocked and the cells survive. Thus, it was concluded that nuclear Ca^{2+} controls the initiation and the progression of PCD in response to sphingolipids (Lachaud et al. 2010).

6 Conclusions and Prospects

The current knowledge of mechanisms underlying Ca^{2+} homeostasis in the nuclei of tobacco cells can be schematically summarized as described in Fig. 2. This very simple cartoon does not take into account the possible regulation of nuclear voltage-sensitive Ca^{2+} channels that could exist in some plant species (legumes) by the activity of cationic channels such as CASTOR and POLLUX, suspected to induce changes in the electrical potential across the inner or outer nuclear membranes (Charpentier et al. 2008; Matzke et al. 2009). Because of the scarcity of available data concerning nuclear Ca^{2+} signaling in plants, our current view of nuclear Ca^{2+} homeostasis is therefore speculative and the hypothesis of nuclear autonomy in terms of calcium management remains to be confirmed by identification and molecular characterization of the expected effectors. Such identification could be achieved by combining genetic and pharmacological approaches. Indeed the use of mutants should confirm or infirm the existence of effectors belonging to the calcium toolkit (Berridge et al. 2000) as for instance putative nuclear iGluR channels involved in

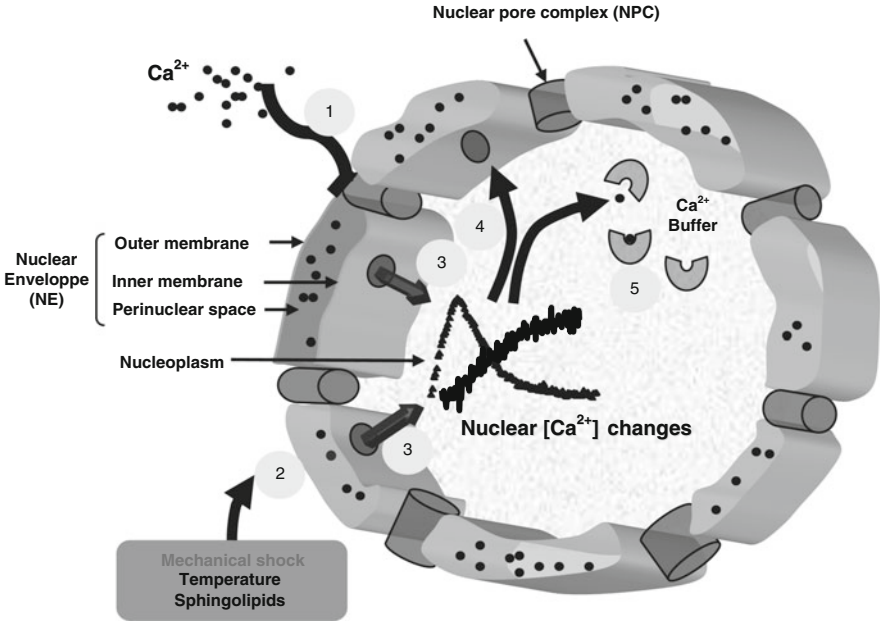

Fig. 2 Model illustrating how isolated plant nuclei can generate $[Ca^{2+}]$ changes and control Ca^{2+} homeostasis. This model was drawn according to published data by Xiong et al. (2004) and Brière et al. (2006) (see text). (1) Ca^{2+} does not diffuse through the NPCs in isolated nuclei whatever the outside $[Ca^{2+}]$. (2) Isolated nuclei can sense chemical (sphingolipids), mechanical or physical (temperature changes) stimuli and generate specific calcium transients. (3) IP_3-dependent and TRP-like channels might be involved in nuclear $[Ca^{2+}]$ variations. (4) Putative Ca^{2+} transporters are predicted by the mathematical model to regulate nucleoplasmic Ca^{2+} concentrations. (5) Nucleoplasmic Ca^{2+}-binding components predicted to buffer nucleoplasmic Ca^{2+} variations

sphingolipid-induced cell death in tobacco BY-2 cells and targeted by pharmacological drugs such as AP5 (Lachaud et al. 2010). The main drawback of these targeted approaches is their poor efficiency in identifying new effectors involved in nuclear Ca^{2+} homeostasis. Thus, a breakthrough will be reached by setting up biochemical methods allowing the isolation of outer and inner membranes from the plant nuclear envelope to perform a global proteomic analysis of each membrane components. One can expect the discovery of new ion transporters or ion channels associated with these membranes from such an approach.

Another improvement of our knowledge of the nuclear calcium role would be to be able to co-localize elementary nuclear Ca^{2+} signals within the numerous nuclear sub-compartments existing in the nucleus as depicted in the poster insert from Spector (2001). Indeed, the eukaryotic nucleus is a highly compartmentalized and dynamic environment (Hager et al. 2009; Misteli 2001; Phair et al. 2004; Spector 2001). Some subdomains have drawn more attention than did others such as the splicing speckles which store the mRNA splicing factors, the nucleolus which is subcompartmentalized by itself and which is involved in ribosomal RNA biogenesis or the Cajal bodies which are very dynamic structures in a permanent assembly/disassembly state and which are involved in various processes such as biogenesis and trafficking of the *s*mall *n*uclear *R*ibo*N*ucleoprotein *P*articles (snRNPs) (Shaw and Brown 2004; Spector 2001). To localize these nuclear Ca^{2+} events, resolution of spatio-temporal detection of calcium signals has to be improved to get a detectable signal in the shortest integration time. This might be achieved through the use of the new generation of cameleon constructs addressed to the nucleus (Sieberer et al. 2009) or through recording the luminescence emitted by the nucleoplasmin–aequorin construct upon Ca^{2+} binding using the forthcoming generation of *E*lectron *M*ultiplying *C*oupled *C*harged *D*evice (EM CCD) cameras technically improved to detect elementary calcium events. Once these calcium signals will be assigned to a specific sub-compartment, the next challenge will be to connect these nuclear Ca^{2+} signals to the nuclear activities associated with these nuclear domains. To date, little is known about the regulatory properties of Ca^{2+} on these events. Thus the expanding field of research devoted to the understanding of how stress responses and plant development can be regulated by the nuclear non-coding RNAs and their interacting RNA-binding proteins (Charon et al. 2010) will open a promising area of research aiming at investigating whether and how they could be regulated by nuclear Ca^{2+}.

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