Signaling and Communication in Plants

Sheng Luan *Editor*

Coding and Decoding of Calcium Signals in Plants

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Editor
Prof. Sheng Luan
University of California, Berkeley
Dept. Plant & Microbial Biology
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94720 Berkeley California
USA

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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

A.J. Trewavas (✉)
Institute of Molecular Plant Science, University of Edinburgh, Rutherford Building, Kings Buildings, Edinburgh EH9 3JH, UK
e-mail: Trewavas@ed.ac.uk

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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

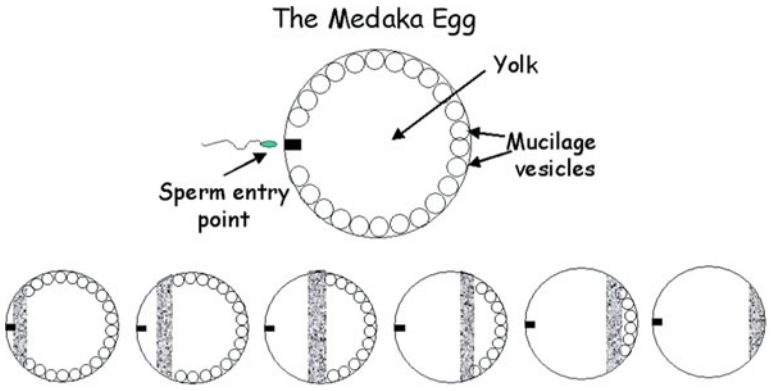

Fig. 1 Selected images of aequorin luminescence (*shaded*) indicating the progress (*from left to right*) of the $[Ca^{2+}]_{cyt}$ wave after fertilisation. Total time = 1 min. Adapted from Gilkey et al. (1978)

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

C. Mazars (✉) • P. Thuleau • V. Cotelle • C. Brière
Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, UPS, UMR 5546, BP 42617, 31326 Castanet-Tolosan, France

CNRS, UMR 5546, BP 42617, 31326 Castanet-Tolosan, France
e-mail: mazars@lrsv.ups-tlse.fr; thuleau@lrsv.ups-tlse.fr; cotelle@lrsv.ups-tlse.fr; briere@lrsv.ups-tlse.fr

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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 *Ca*l*M*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

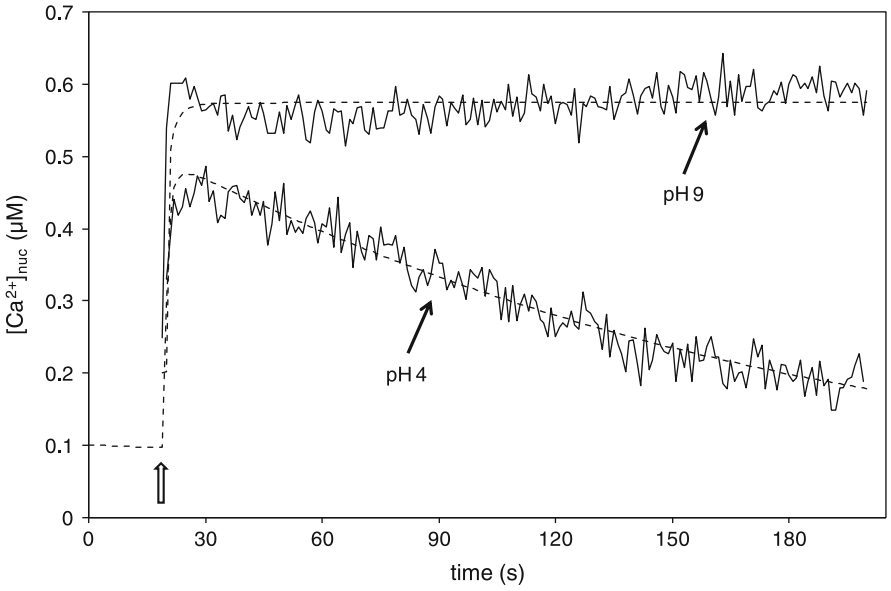

Fig. 1 Calcium variations induced by mechanical stimulation of isolated nuclei at two pH values. *Broken line*: adjustment of the model from Brière et al. (2006) to experimental data (Xiong et al. 2004) (*continuous line*). At acidic pH a mechanical stimulus induces a transient influx of calcium within 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

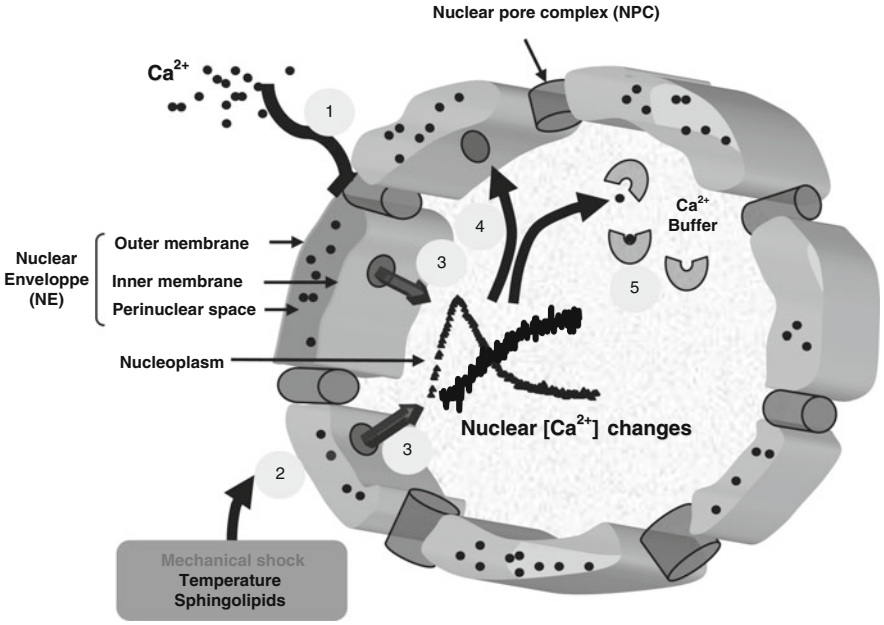

Fig. 2 Model illustrating how isolated plant nuclei can generate $[Ca^{2+}]$ changes and control Ca^{2+} homeostasis. This model was drawn according to published data by Xiong et al. (2004) and Brière et al. (2006) (see text). (1) Ca^{2+} does not diffuse through the NPCs in isolated nuclei whatever 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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Interactions Between Calcium and ROP Signaling Regulate Pollen Tube Tip Growth

Augusta Jamin and Zhenbiao Yang

Abstract The pollen tube has long been a favorite system to investigate the regulation and function of the spatiotemporal calcium signal in plants. Calcium is critical for polarized cell growth in pollen tubes, termed tip growth, and its intracellular accumulation at the tip region of pollen tubes displays a tip-high gradient and an oscillatory pattern that is tightly correlated with growth oscillation. One calcium oscillator in pollen tube is a ROP GTPase, which belongs to a conserved Rho family of small GTPases. Active ROP1 oscillates in a phase ahead of growth oscillation, and the distribution of active ROP1 to the apical plasma membrane defines tip growth site and is required for pollen tube growth. This polarized growth depends on many factors including active ROP1-regulated downstream factors: F-actin dynamics and tip-focused calcium gradients. These downstream elements are in turn involved in positive and negative feedback regulations of ROP1, respectively. In addition, ROP1 activity is regulated both spatially and temporally by several regulatory proteins, which may be regulated by calcium.

1 Introduction

In flowering plants, pollen tubes play a major role in the fertilization and reproductive process. Pollen grains land on the stigma surface of the female tissues and germinate to form pollen tubes. Pollen tubes grow within the pistil and are attracted

A. Jamin • Z. Yang (✉)
Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA
e-mail: yang@ucr.edu

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to the ovules where tubes then burst open to release the male gametes that fertilize the egg and central cells (Palanivelu and Preuss 2006; Stewman et al. 2010). Pollen tubes undergo tip growth, during which cell membrane growth and wall expansion is restricted to the tip region where signaling processes occur to regulate growth (Cheung and Wu 2008; Lee and Yang 2008; Yang 2008). Pollen tube growth is known to be regulated by several signaling processes including ROP GTPase signaling pathways, ionic regulation such as spatiotemporal calcium (Ca^{2+}) signaling, lipid-dependent signaling pathways, and vesicular trafficking and cell wall modification (Berken 2006; Cole and Fowler 2006; Krichevsky et al. 2007; Cheung and Wu 2008; Lee and Yang 2008; Yang 2008; Zonia 2010). ROP belongs to the Rho family of small GTPases and its role has been linked to cell polarity regulation through the control of cytoskeletal dynamics and vesicular trafficking (Yang 2008; Nibau et al. 2006; Brembu et al. 2006; Winge et al. 2000; Vernoud et al. 2003). ROP1 activation and regulation are required for normal pollen tube growth. Disruption of ROP1 leads to severe growth defect. Overexpression (OX) of ROP1 or its constitutive-active form (CA-rop1) resulted in swollen tubes while its dominant-negative form (DN-rop1) resulted in short tubes (Li et al. 1999; Kost et al. 1999).

Pollen tube growth also requires a Ca^{2+} gradient in the apical region of the cytoplasm (Li et al. 1999; Holdaway-Clarke et al. 1997; Pierson et al. 1994, 1996). Accumulation of tip-Ca^{2+} through Ca^{2+} influx is regulated by the ROP1 pathway specifically via its downstream effector, ROP-interactive CRIB-containing protein 3 (RIC3) (Gu et al. 2005). Disruption of Ca^{2+} gradient by manipulating RIC3 levels, via injecting with anti-ROP1P antibody or treating pollen tubes with Ca^{2+} chelator, Ca^{2+} ionophore, or caffeine, affected pollen tube growth (Gu et al. 2005; Pierson et al. 1994, 1996; Lin and Yang 1997; Li et al. 1999; Yan et al. 2009). Tip-focused Ca^{2+} has an indirect antagonistic relationship with F-actin assembly regulated by another ROP1 effector, RIC4, and is likely to promote actin disassembly by acting on actin regulators such as profilin (Gu et al. 2005). Recent evidence suggests that Ca^{2+} is involved in the negative feedback regulation of ROP1, though the mechanism by which Ca^{2+} participates in this negative feedback regulation remains to be elucidated (Yan et al. 2009).

Oscillatory signaling pathways have been observed in several biological systems, e.g., circadian oscillator in cyanobacteria (Dong and Golden 2008), plants (Harmer 2009), *Neurospora*, *Drosophila*, and mammalian systems (Dunlap 1999; Jolma et al. 2010). Interestingly, ROP1 activation at the tip of pollen tubes oscillates with a similar period as but ahead of growth oscillation (Hwang et al. 2005). In addition, tip-focused Ca^{2+} oscillates in phase with growth oscillation, but Ca^{2+} influx oscillates behind growth oscillation (Holdaway-Clarke et al. 1997). Based on these oscillation data, phase relationships between active ROP1, tip-focused Ca^{2+}, and tip growth can be drawn; however, the question still remains as to what factors initiate these oscillations.

2 Pollen Tube As a Model System for Tip Growth and Growth Oscillation

Tip growth in plant cells was first reported over 50 years ago, and tip growth is known to result from the addition of new cell wall materials and the displacement of older wall materials at the growing region of the cell (Castle 1958). As a dynamic single-cell system, pollen tube is a valuable system to study tip growth. Pollen tubes are easily cultured *in vitro* and the dynamic cellular imaging can be easily performed without damaging its cellular integrity (Fu et al. 2001; Gu et al. 2005; Hwang et al. 2005; Lee et al. 2008). Molecular tools and techniques for transient or stable expressions are widely available such as pollen-specific promoter Lat52 (Twell et al. 1991; Eyal et al. 1995), particle bombardment for plasmid delivery into pollen tubes (Twell et al. 1989), and microinjection for the delivery of Ca^{2+} indicators or buffers (Pierson et al. 1994; Camacho et al. 2000), or antibodies (Lin and Yang 1997). In addition, pollen tubes maintain certain shapes and growth patterns such that deviation from normalcy can be easily scored. Most importantly, due to its haploid genome, lethal mutations which affect pollen tube development or growth are transmissible through heterozygous plants.

The growth rates of pollen tubes vary depending on the species. *Arabidopsis*, tobacco, and lily pollen tubes can grow with rates such as micrometers per minute while maize pollen tubes grow at faster rates of sub-millimeters per minute (Holdaway-Clarke et al. 1997; Hwang et al. 2005; Iwano et al. 2009; House and Nelson 1958; Messerli and Robinson 1997). In spite of the variability in growth rates, a common feature among these tip growing cells is their oscillatory growth. Biological oscillation has been observed in many biological systems; yet it is still unclear as to how biological oscillation arises (Goldbeter 2002). One idea is that biological interactions of cellular components within a cell may spontaneously produce oscillatory behaviors (Feijo et al. 2001). Thus, oscillations may be obtained based on positive feedback alone, negative feedback alone (Goldbeter 2002), or both positive and negative feedback loops to create a more robust and tunable frequency (Tsai et al. 2008). In the end, the implication of an oscillatory system is essentially the production of a biological system with self-organizing processes (Feijo et al. 2001).

Pollen tubes are particularly suited for dissecting sub-cellular oscillatory behaviors at high spatial and temporal resolutions, because of their short oscillation frequencies, which facilitates microscopic imaging of sub-cellular events and activities. Pollen tubes from both lily and tobacco display oscillatory tip growth with mean period of 30–42 s (growth rate = 0.1–0.38 μm/s) and 80 s (growth rate = 25 nm/s), respectively (Holdaway-Clarke et al. 1997; Messerli and Robinson 1997; Pierson et al. 1996; Hwang et al. 2005). In *Arabidopsis* pollen tubes, however, growth oscillation appears to occur with a frequency of 20 s and a slower growth rate of 33 nm/s (Iwano et al. 2009).

3 Ca^{2+} Oscillation in Pollen Tubes

Ca^{2+} is essential for normal pollen tube growth. Studies using Ca^{2+} dyes showed steep Ca^{2+} gradient around 1 μM to 1 mM at the tip region and basal Ca^{2+} level around 150–300 nM at the subapical region (20 μM from the tip) of lily pollen tube (Pierson et al. 1994, 1996; Holdaway-Clarke et al. 1997; Camacho et al. 2000). The steep tip-focused Ca^{2+} gradient has also been observed in *Agapanthus*, pea, silverworts (*Tradescantia*), tobacco, and *Arabidopsis* (Malho et al. 1994; Li et al. 1999; Pierson et al. 1996; Iwano et al. 2009). The importance of tip-localized Ca^{2+} in pollen tube growth was suggested by several studies. Dissipation of tip-localized Ca^{2+} using Ca^{2+} chelator BAPTA-type buffers, caffeine, and mild thermal shock inhibited pollen tube elongation (Pierson et al. 1994, 1996). In addition, pollen germination and tube elongation is impaired at extra low (<0.5 mM) or high (>10 mM) levels of extracellular Ca^{2+} and is optimal at medium levels (2–5 mM) (Li et al. 1999; Gu et al. 2005). The mechanisms underlying growth inhibition in low or high Ca^{2+} levels are still unclear. Growth inhibition in low Ca^{2+} is likely due to less Ca^{2+} influx at the tip region, which results in lower tip-localized Ca^{2+} accumulation (Pierson et al. 1994; Li et al. 1999). In contrast, growth inhibition by high Ca^{2+} may be due to negative feedback regulation of Ca^{2+} influx (Li et al. 1999) or due to Ca^{2+}-mediated increase in cell wall rigidity caused by cross-linking of Ca^{2+} to the apical pectic wall (Holdaway-Clarke et al. 1997).

The maintenance of a proper intracellular Ca^{2+} level is essential for normal pollen tube growth and may involve Ca^{2+} pumps and/or channels. A class of Ca^{2+} pumps, autoinhibited Ca^{2+} ATPases (ACA) family members such as ACA9, is required for normal pollen tube growth and fertilization since *aca9* knockout had reduced tube growth as well as reduced frequency of tubes reaching the egg cells (Schiott et al. 2004). The molecular mechanisms underlying ACA9 regulation of Ca^{2+} signaling in pollen tube is unclear. It is possible that ACA9 functions to maintain Ca^{2+} homeostasis or that it is a part of Ca^{2+} oscillator by providing a regulated efflux pathway (Schiott et al. 2004). More recently, a Ca^{2+} channel, cyclic nucleotide-gated channel (CNGC) 18 was shown to be important for pollen tube growth (Frietsch et al. 2007). *cngc18-1* pollen tubes were short, kinky, had non-directional growth, and often burst. In addition, these tubes failed to grow within the female tissues in vivo (Frietsch et al. 2007). CNGC18 appears to be a Ca^{2+} permeable channel (Frietsch et al. 2007). However, CNGC18 is permeable to other ions including K^+ (Frietsch et al. 2007), and thus it is unclear whether it is the major Ca^{2+} channel responsible for Ca^{2+} influx at the tip of pollen tubes. Overall, the lack of well-characterized Ca^{2+} channels has limited our understanding of their roles in regulating intracellular Ca^{2+} levels. Future findings of additional Ca^{2+} transporters/channels should enhance our understanding of Ca^{2+} signaling in the pollen tube system.

A positive correlation exists between tip-focused Ca^{2+} and growth rate such that low intracellular Ca^{2+} is associated with slower growth while high intracellular Ca^{2+} with faster growth (Pierson et al. 1996). In lily pollen tubes, intracellular

tip-localized Ca^{2+}, measured using Ca^{2+} indicator fura-2-dextran, oscillates in phase and with similar period as growth oscillation (Holdaway-Clarke et al. 1997; Messerli and Robinson 1997; Pierson et al. 1996). In contrast, apparent extracellular- Ca^{2+} influx oscillates in a similar period but behind growth oscillation by 60° (Holdaway-Clarke et al. 1997). To explain the time delay observed between the oscillations of extracellular-Ca^{2+} influx or tip-localized Ca^{2+}, and growth, Holdaway-Clarke et al. proposed two possible mechanisms. First, the release of Ca^{2+} from internal stores such as the ER or vesicles may promote exocytosis and subsequently cell elongation. Second, as cell elongates, pollen tube cell wall at the tip region is continually modified by pectin methylesterases (PMEs), which catalyze the cleavage of methylester groups from the pectin and leave the acidic residues exposed (Holdaway-Clarke et al. 1997; Bosch et al. 2005). This results in the crosslink between free Ca^{2+} and the deesterified pectin and eventually would slow pollen tube growth due to reduced elasticity of cross-linked pectin. Thus the time that it takes for PMEs to act on its pectin substrate as well as the diffusion of free Ca^{2+} onto the cell wall space may be the reason as to why time delay was observed between the oscillation of extracellular-Ca^{2+} influx and growth (Holdaway-Clarke et al. 1997).

4 ROP Signaling in Regulating Pollen Tube Tip Growth

ROP was first cloned from garden pea (Yang and Watson 1993) and has since been identified and/or characterized in various plants such as *Arabidopsis* (Li et al. 1998; Winge et al. 1997), tobacco (Klahre et al. 2006), *Brassica napus* (Chan and Pauls 2007), rice (Chen et al. 2010), and moss (Eklund et al. 2010). In *Arabidopsis*, three closely related ROPs (ROP1, 3, and 5) are expressed in pollen and are functionally redundant in the regulation of pollen tube growth, although ROP1 is pollen-specific and most extensively studied (Li et al. 1998, 1999). Consistent with its role in the regulation of polarized tip growth, ROP1 is found to localize to the apical region of pollen tubes (Lin et al. 1996; Hwang et al. 2008). The apical domain of active ROP1 is thought to be the region of cells where growth takes place. Overexpression of ROP1 or its tobacco homolog NtRac5 resulted in wider tubes and swollen tips, which can be correlated with the expansion of ROP1 domain at the apical region (Li et al. 1999; Klahre et al. 2006; Hwang et al. 2010). CA-rop1 and CA-NtRac5, which contains mutation (Gly15) that locks the protein in GTP-bound active form, when overexpressed, produced pollen tubes that were severely depolarized (Li et al. 1999; Klahre et al. 2006). In contrast, DN-rop1 and DN-NtRac5, which contains mutation (Asp121) that locks the protein in the GDP-bound inactive form, when overexpressed, caused inhibition of tube growth (Li et al. 1999; Kost et al. 1999).

Active ROP1 at the apical cap is generated from tip-localized ROP1 activation that laterally spread within the apical region (Hwang et al. 2010, 2008). This lateral propagation is likely due to the *de novo* activation of ROP1 rather than the stabilization of active ROP1 at the apical PM (Hwang et al. 2010). Active ROP1

at the apex of pollen tube regulates tip growth (Hwang et al. 2005, 2010). One mechanism by which active ROP1 controls tip growth is through the modulation of tip-targeted exocytosis (Lee et al. 2008). Using a fluorescence recovery after photobleaching (FRAP)-based method to visualize exocytosis, Lee et al. showed that DN-rop1 inhibited exocytosis to the growth site, while CA-rop1 depolarized the site of exocytosis (Lee et al. 2008).

5 Downstream Effectors of ROP1 and Ca^{2+} Signaling

Two ROP-interactive CRIB-containing (RIC) proteins have been shown to act as ROP1 effector proteins that are involved in regulating pollen tube tip growth (Gu et al. 2005). Both RIC3 and RIC4 interact with ROP1 at the tip region of pollen tubes *in vivo* and their overexpression induced depolarization of pollen tube similar to that caused by ROP1 OX (Gu et al. 2005). Disruption of RIC3 and RIC4 localization to the tip region by mutating two conserved histidine residues (His37 and His40) in the CRIB motif abolished the RIC-induced tip swelling phenotype (Gu et al. 2005).

RIC4 is involved in F-actin dynamics by promoting actin assembly. This is evident from RIC4 OX, which resulted in a dense F-actin network at the tip region as well as disruption of tip F-actin oscillation (Gu et al. 2005). The effects of RIC4 OX were reversed by LatB treatment, which promotes F-actin depolymerization (Fu et al. 2001). In contrast, RIC3 is involved in regulating tip-localized Ca^{2+} gradients. *ric3RNAi* inhibited tube growth in the presence of moderate extracellular Ca^{2+} levels (2–5 mM), but promoted growth in the presence of high extracellular Ca^{2+} levels (10 mM) (Gu et al. 2005). On the contrary, RIC3 OX promoted tube growth in low Ca^{2+} level (0.5 mM) but inhibited growth at medium Ca^{2+} level (>2 mM); and this growth inhibition can be restored by $LaCl_3$ treatment, which blocks Ca^{2+} influx (Gu et al. 2005). Further examination of intracellular Ca^{2+} accumulation supports the hypothesis that RIC3 promotes Ca^{2+} influx at the tip, as evident from the observation that RIC3 OX tubes grown in 100 μM extracellular Ca^{2+} displayed wild type-like tip-focused Ca^{2+} but in 1 mM Ca^{2+} displayed expanded and more intense tip-focused Ca^{2+} compared to wild type (Gu et al. 2005). Consistently, blocking ROP1 signaling by injection of ROP1 antibody inhibited tube elongation as well as diminished tip-focused Ca^{2+} (Lin and Yang 1997; Li et al. 1999). Thus, ROP1 directly regulates the formation of tip-localized Ca^{2+} gradients through the action of RIC3.

RIC4 and RIC3 have an indirect antagonistic relationship through their downstream targets. RIC3 OX induced actin reorganization at the tip region, which includes loss of tip F-actin and protrusion of actin cables to the apical region (Gu et al. 2005). Disruption of tip F-actin due to RIC3 OX involves tip-focused Ca^{2+} since Ca^{2+} chelator, EGTA, or Ca^{2+} channel blocker, $LaCl_3$, can suppress the RIC3 OX-induced tip F-actin disorganization (Gu et al. 2005). In addition, RIC3 OX can indirectly suppress the effects of RIC4 OX via Ca^{2+}, which affects actin

dynamics likely by activating actin disassembly factors such as profilin (Gu et al. 2005). In contrast, suppression of tip-localized Ca^{2+} by RIC4 involves F-actin assembly acting on a Ca^{2+} signaling pathway (Gu et al. 2005).

Tip F-actin regulated by RIC4 has a role in exocytosis process by promoting the accumulation of exocytic vesicles to the tip region. By using an exocytic vesicle marker *YFP-RabA4d*, Lee et al. showed that apical F-actin is required for tip accumulation of YFP-RabA4d to the apical clear zone marked by an inverted cone shape localization, which represents exocytic vesicles. Apical F-actin stabilization caused by RIC4 OX induced vesicles accumulation to the apical cortex, whereas disassembly caused by RIC3 OX resulted in the loss of YFP-RabA4d localization to the apical region (Lee et al. 2008). Consistently, $LaCl_3$ can recover the YFP-RabA4d localization in the RIC3 OX background while LatB or RIC3 coexpression can recover the YFP-RabA4d localization in the RIC4 OX background (Lee et al. 2008).

6 Regulation of ROP1 in Pollen Tubes

The dynamics of ROP1 activity is critical for polarized tip growth, suggesting an elaborate spatiotemporal regulation of the apical ROP1 activity (Hwang et al. 2005, 2010). To understand this regulation, it is important to characterize factors that regulate ROP1 activity. Three types of ROP regulatory proteins have been characterized. Guanine dissociation factor (GDI) is known to negatively affect ROP1 association to the PM by sequestering ROP1 within the cytosol (Klahre et al. 2006; Hwang et al. 2010). NtRhoGDI2 OX inhibited pollen tube growth but co-expression with NtRac5 suppressed NtRac5 OX-induced tip swelling and produced normal pollen tube (Klahre et al. 2006). Similar observation was also reported for AtRhoGDI1 suppression of AtROP1 OX-induced tip swelling by limiting the lateral propagation of apical ROP1 (Hwang et al. 2010). Consistently co-expression of NtRhoGDI2 with DN-NtRac5 further inhibited pollen tube growth while NtRhoGDI2 with CA-NtRac5 had no effect on tube swelling (Klahre et al. 2006). No effect on tube swelling was also reported for AtRhoGDI1 co-expression with AtCArop1 (Hwang et al. 2010). Based on its cytosolic localization and its action in counteracting apical ROP1 activation, RhoGDI1 can be considered as a global inhibitor of apical ROP1 (Hwang et al. 2010).

Positive regulatory protein, guanine nucleotide exchange factor (RopGEF), catalyzes GDP to GTP exchange, which results in the activation of ROP1. In *Arabidopsis*, 14 RopGEFs have been identified (Berken et al. 2005; Gu et al. 2006). RopGEF1 OX induced depolarized growth similar to CA-rop1 phenotype (Gu et al. 2006). Co-expression of RopGEF1 with DN-rop1 suppressed the RopGEF1 OX-induced growth depolarization, which suggests that RopGEF1 activity requires GTP-bound ROP1 (Gu et al. 2006). GEF activity assay using deletion mutants of RopGEF1 and ROP4 as a substrate showed that the three conserved domains, C1–C3, contain GEF activity and thus were named the PRONE

(plant-specific Rop nucleotide exchanger) domain (Berken et al. 2005). The variable C-terminal region of RopGEF1 was found to auto-inhibit the GEF activity of RopGEF1 by interacting with the PRONE/DUF315 domain (Gu et al. 2006). Another report suggests that inhibition of AtRopGEF12 by its C-terminal region can be released by phosphorylation possibly involving pollen receptor kinase, AtPRK2a (Zhang and McCormick 2007). This phosphorylation potentially releases the N-terminal region and thus allows ROP1 to interact with the PRONE domain (Zhang and McCormick 2007). This is further supported by the findings that OX of wild type AtRopGEF12 alone did not induce growth depolarization, but co-expression of AtRopGEF12 and AtPRK2a or phosphorylation-mimic AtRopGEF12 mutant alone resulted in severe depolarized growth (Zhang and McCormick 2007).

GTPase activating protein (RopGAP) promotes GTP hydrolysis, leading to ROP1 inactivation. In *Arabidopsis*, five RopGAPs have been identified (Wu et al. 2000). Co-expression of one particular RopGAP, RopGAP1, with ROP1 resulted in the suppression of ROP1 OX-induced tip swelling phenotype by limiting ROP1 activation at the apical PM (Hwang et al. 2010). This suppression of ROP1 depends on RopGAP's catalytic activity since catalytic mutation at Arg202 renders the protein inactive (Hwang et al. 2010). Consistently, overexpression of NtRhoGAP1 induced short tubes with narrower tips (Klahre and Kost 2006). In addition to the GAP domain, RopGAP proteins also contain Cdc42/Rac-interactive binding (CRIB) motif which itself does not possess any GAP activity but its presence enhances GAP activity in RopGAP1 by promoting binding of RopGAP1 and ROP1 (Wu et al. 2000). Similar observation has been reported in tobacco NtRhoGAP1 whose GAP activity toward NtRac5 is enhanced in the presence of CRIB motif (Klahre and Kost 2006). NtRhoGAP1 was localized to the shoulder of the apical PM (Klahre and Kost 2006), but RopGAP1 was localized to the apical PM (Hwang et al. 2010). It is possible that these two RopGAPs may have different functions in the regulation of ROPs in the pollen tube tip.

A new class of GAP proteins that is distinct from RopGAPs was recently identified (Hwang et al. 2008). *Rop enhancer 1 (REN1)* encodes a novel RhoGAP containing pleckstrin homology (PH) domain, GAP domain, and C-terminal coiled coil region (Hwang et al. 2008). Loss of function *ren1-1* resulted in severely depolarized tubes similar to CA-rop1 phenotype, and consistent with the phenotype, ROP1 localization at the apical region was expanded in the *ren1-1* background, suggesting its role in the spatial regulation of ROP1 (Hwang et al. 2008). REN1 is localized to the apical region as well as in exocytic vesicles, and tip-directed targeting of REN1 via exocytosis is required to restrict ROP1 activity at the tip region (Hwang et al. 2008). Tip-localized REN1 oscillates with similar period but behind of active ROP1 oscillation by 60° while it oscillates ahead of growth oscillation by 30° (Hwang et al. 2008). It was proposed that ROP1 activation promotes tip-directed targeting of REN1 to the apical region via exocytic vesicles, where it then inactivates ROP1 and eventually slows pollen tube growth (Hwang et al. 2008).

7 Oscillation of ROP1 and Its Effectors in Relations to Oscillation of Tip-Focused Ca^{2+}

As a downstream effector, which localizes to the apical PM and interacts with active ROP1, RIC4 can be engineered as a marker to report active ROP1 in oscillating pollen tubes (Gu et al. 2005; Hwang et al. 2005). RIC4 OX resulted in depolarized pollen tube growth and thus is not a useful tool as a ROP1 marker. However, C-terminal deletion of RIC4 (RIC4ΔC) abolished effector's activity without affecting its interaction with active ROP1 (Hwang et al. 2005). When overexpressed, GFP-RIC4ΔC did not affect pollen tube growth. By using GFP-RIC4ΔC as an active ROP1 marker, it was observed that active ROP1 at the apical PM oscillates with a mean period of 60–70 s and a phase that is 90° ahead of growth oscillation (Hwang et al. 2005; Hwang et al. 2010). Similarly, tip-localized F-actin oscillates 70° ahead of growth oscillation (Hwang et al. 2005; Fu et al. 2001). This observation raises the possibility that actin dynamics may be important for ROP1 activity and/or oscillation in pollen tube growth regulation. This was supported by the observation that changing F-actin dynamics by LatB in GFP-RIC4 OX tubes recovered growth oscillation (Hwang et al. 2005). Ca^{2+} influx and the maintenance of tip-focused Ca^{2+} gradient may also be required for ROP1 activity and growth oscillation since $LaCl_3$ treatment of GFP-RIC4 tubes resulted in reduced growth rate, increased oscillation period, and reduced oscillation amplitude (Hwang et al. 2005).

A mathematical model was described that simulates the interaction between the RIC4-mediated F-actin pathway and the RIC3-mediated Ca^{2+} pathway. The model involves two interlinked feedback loops that are essential for ROP1 activity oscillation (Fig. 1a). One loop consists of RIC4-dependent F-actin in the positive feedback pathway, and another loop consists of RIC3-Ca^{2+} in the negative feedback regulation of ROP1 (Yan et al. 2009). This model simulated the phase relationships between ROP1 activity, F-actin, and Ca^{2+} in the oscillation (Fig. 1b). The simulation predicted that the stabilization of F-actin increased ROP1 activity and diminished ROP1 oscillation (Yan et al. 2009). This prediction was validated by experimental data showing that actin stabilization by jasplakinolide treatment caused an increase in the apical ROP1 activity and dampening of both growth oscillation and active ROP1 oscillation. The model also predicted that the depletion of F-actin would reduce ROP1 activity and diminish ROP1 oscillation, which is consistent with experimental evidence that showed reduced amplitude of active ROP1 oscillation in GFP-RIC4ΔC pollen tubes treated with 0.5 nM LatB and restored F-actin oscillation in ROP1 OX tubes treated with 5 nM LatB (Yan et al. 2009; Hwang et al. 2005; Fu et al. 2001).

In the negative feedback loop, Ca^{2+} negatively regulates ROP1 activity by either promoting F-actin disassembly and thus counteracts the F-actin-mediated positive feedback loop or by activating negative regulators of ROP1 (Yan et al. 2009). In either case, oscillation of both active ROP1 and free Ca^{2+} can be simulated granted that there is appropriate time delay of 8 s between ROP1 activation and

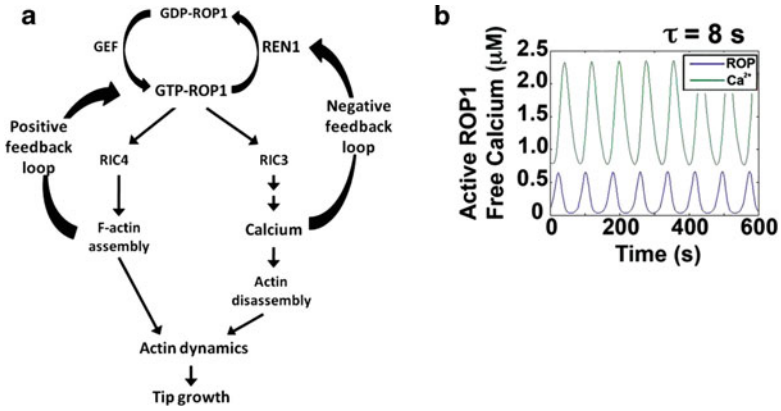

Fig. 1 Computational modeling of the mechanism underlying ROP1 activity oscillation. (**a**) The structure of the mathematical models for two feedback loops regulating ROP1 activity in pollen tubes. Positive feedback loop involves RIC4 that promotes F-actin accumulation, which positively regulates ROP1 activation, while negative feedback loop involves either Ca^{2+} acting on actin depolymerizating factors to promote actin disassembly or on the REN1 RhoGEF. (**b**) Simulation of the active ROP1 and free calcium oscillation based on the models described in (**a**) (Yan et al. 2009)

tip-Ca^{2+} accumulation (Yan et al. 2009). Simulation showed that increase in Ca^{2+} accumulation to a high level led to the suppression of ROP1 activity and the loss of ROP1 oscillation, while a moderate increase in Ca^{2+} accumulation led to a moderate reduction in ROP1 activity with lower amplitudes on ROP1 oscillation (Yan et al. 2009). Consistent with this simulation, high Ca^{2+} accumulation caused by either Ca^{2+} ionophore, A23187, or RIC3 OX resulted in reduced apical ROP1 accumulation (Yan et al. 2009).

8 A Working Model for Ca^{2+} Regulation of ROP1 Oscillation

Tip-focused Ca^{2+} gradients in pollen tubes were discovered over 30 years ago, and its essential role in the regulation of pollen tube tip growth has also been well-recognized (Jaffe et al. 1975; Weisenseel et al. 1975; Rathore et al. 1991). However, their mode of action is still poorly understood. A recent study suggesting Ca^{2+}'s role in the negative feedback loop in regulating ROP1 activity provides some new insights into the Ca^{2+}'s mode of action in pollen tubes (Yan et al. 2009). Interestingly, circumstantial evidence supports a functional interaction between Ca^{2+} and REN1 (Hwang et al. 2008). Pollen tube growth in weak *ren1* alelle (*ren1-3*) was normal at medium Ca^{2+} level (2–5 mM) but became depolarized under low extracellular Ca^{2+} condition (0.5 mM), suggesting that REN1 and Ca^{2+} may work together in the REN1-based negative feedback loop (Hwang et al. 2008).

We speculate that Ca^{2+} may act through a Ca^{2+} sensor to regulate REN1 activity in this negative feedback loop.

A good candidate for the Ca^{2+} sensor in the proposed REN1 regulation is Ca^{2+}-dependent protein kinase (CDPK/CPK), which has been linked to the regulation of normal pollen tube growth (Harper et al. 2004). This class of Ca^{2+} sensors is unique compared to the other known Ca^{2+} sensors in eukaryotes since its activity requires only Ca^{2+} but not calmodulin or phospholipids (Harmon et al. 1987; Harper et al. 1991). Structurally, CPKs consist of a kinase domain, autoinhibitory region, and EF-fingers calmodulin-like Ca^{2+}-binding motifs (Harper et al. 1991; Huang et al. 1996). When the Ca^{2+} level is below a certain threshold, the autoinhibitory region interacts with the kinase domain and renders the protein kinase inactive. Increase in Ca^{2+} levels (above a certain threshold), which is detected by the Ca^{2+}-binding motifs, leads to the release of the kinase domain (Huang et al. 1996; Yoo and Harmon 1996; Lee et al. 1998). A study by Moutinho et al. using fluorophore-tagged bisindolylmaleimide, a kinase inhibitor selective for protein kinase C, implies the presence of a tip-high Ca^{2+}-dependent kinase activity in growing tubes of *Agapanthus* (Moutinho et al. 1998). This kinase activity might be encoded by a CPK.

In *Arabidopsis*, there are 34 CPK isoforms, 12 of which show significant pollen expression (Becker et al. 2003; Myers et al. 2009). Zhou et al. reported that CPK32 OX resulted in swollen tubes, as does ROP1 OX, suggesting that CPK may be involved in the regulation of ROP1 in response to Ca^{2+} (Zhou et al. 2009). Another pollen-expressed CPK17, and its homolog CPK34, have also been shown to be critical for normal pollen tube growth (Myers et al. 2009). In yet another study, DN-PiCDPK1 OX from petunia resulted in tip swelling phenotype, whereas CA-PiCDPK1 OX inhibited tube growth similar to that induced by DN-rop1 OX (Yoon et al. 2006). These observations are consistent with the notion that CPK is a negative regulator in ROP1 signaling. Therefore, it is tantalizing to speculate that CPK senses high levels of tip Ca^{2+} and promotes REN1 activation in the negative feedback loop.

9 Concluding Remarks

Studies have established a complex connection between intracellular tip-focused Ca^{2+} gradient and ROP GTPases signaling in the regulation of pollen tube growth. The formation of the tip-focused Ca^{2+} gradients is regulated by one branch pathway of ROP1 signal – the RIC3 pathway. RIC3-mediated Ca^{2+} promotes actin depolymerization to antagonize the RIC4 pathway that promotes actin polymerization. Ca^{2+} also feedback-regulates the activity of ROP1 probably by modulating ROP1 regulators such as REN1 (RhoGAP). These findings have also raised important questions needing further investigation. It is unknown how RIC3 promotes the formation of the tip-focused Ca^{2+} gradients. One major challenge in addressing this question is the lack of molecular identities of Ca^{2+} transporters that are responsible

for the Ca^{2+} gradient formation. Another major future issue is to determine Ca^{2+} sensors that link Ca^{2+} to pollen tube tip growth and feedback regulation of ROP1. One likely series of candidate Ca^{2+} sensors that could make these connections is pollen-expressed CPKs (Zhou et al. 2009). Investigating the roles of CPKs and their connections to ROP signaling should be an exciting research area in the future.

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Calcium, Mechanical Signaling, and Tip Growth

Won-Gyu Choi, Sarah J. Swanson, and Simon Gilroy

Abstract Changes in cytosolic Ca^{2+} have emerged as important regulators of plant growth. During tip growth, changes in cytosolic Ca^{2+} appear to trigger proton fluxes and reactive oxygen species (ROS) production to the apoplast of the growing cell, likely to reinforce the wall to prevent uncontrolled expansion. In addition, ROS permeate to the cytosol to act as signals triggering a range of downstream processes, including Ca^{2+} channel gating. Thus, in a complex feedback process, the extent of the Ca^{2+} gradient at the growing tip is modulated to precisely control growth. These changes bear striking similarities to responses elsewhere in the plant to physical stimuli, where Ca^{2+}, ROS, pH also all play roles in the mechanoresponse. Analyses of Ca^{2+}-responsive signaling elements such as calmodulin and the CDPKs is beginning to reveal how such Ca^{2+} changes may be decoded to control growth. Networks of these Ca^{2+}-response pathways tuned to "listen" to particular components of the Ca^{2+} signal may help explain how plants can so exquisitely integrate and entrain their responses to current environmental conditions to effect plastic development.

1 Introduction

The sessile nature of their lifestyle means that plants must sense their environment and mount the appropriate biochemical, physiological, and developmental responses to adapt to ever-changing surroundings. Despite the many differing stimuli such as light direction and quality, water availability, nutrient levels, and biotic and abiotic stresses that provide key clues to the plant as to its current environment, calcium is recognized as a common regulator of such responses.

W.-G. Choi • S.J. Swanson • S. Gilroy (✉)
Department of Botany, University of Wisconsin, Birge Hall, 430 Lincoln Drive, Madison, WI 53706, USA
e-mail: sgilroy@wisc.edu

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For example, Ca^{2+} signals are known to be involved in triggering processes as diverse as the production of biochemical defenses to pathogens, and the modulation of gene expression related to tolerating cold stress (Dodd et al. 2010; Kudla et al. 2010). A major component of plant-adaptive success lies in each plant's ability to tailor its growth and development to ambient conditions, so-called "plastic" development. Here too, evidence has amassed implicating Ca^{2+} as a central regulatory molecule intimately involved in triggering response pathways. The involvement of this ion has been implicated at all levels from the signaling linking the perception machinery for environmental stimuli (such as the direction of light; Harada and Shimazaki 2007), to the response/coordination machinery acting on this information (such as hormonal signaling pathways, Kim et al. 2010) through to the cellular machinery triggering growth (Cardenas 2009) and developmental pathways (Richter et al. 2009). In this chapter we will explore some of this evidence for Ca^{2+} as a key modulator of cell growth and discuss how it could play an important role in driving the plastic nature of plant development.

2 Ca^{2+} and Tip Growth

Polarized cells such as root hairs, pollen tubes, fungal hyphae, and algal rhizoids have provided extremely useful models in which to probe how Ca^{2+} may act to modulate cell expansion. In these cells, the incorporation of new membrane and cell wall components is limited to a very small region at the tip of the expanding cell, leading to a long tube-like growth habit. Such tip growth is associated with a tip-focused gradient in Ca^{2+} that drives polarized secretion as well as helping to maintain the distribution of the cytoskeleton, organelles, and biochemical activities that support the polar character of elongation (Cheung and Wu 2008).

The localized nature of this Ca^{2+} gradient is thought to be maintained by two opposing activities: (1) localized, tip-focused gating of Ca^{2+} channels driving the increase in Ca^{2+} levels in the cytosol and (2) pumps and Ca^{2+} sequestration mechanisms limiting the spread of this increase only to the extreme apex. Ca^{2+} is a relatively immobile ion in the cytosol and so a combination of local influx and sequestration/removal can sustain an extremely steep Ca^{2+} increase limited to the apex. This is an important feature as a sustained, elevated cytosolic Ca^{2+} level above the approximately 100 nM basal concentrations achieved in most cells proves to be cytotoxic through processes such as precipitation with phosphate within the cell. By maintaining an extremely tight spatial and temporal control of this gradient, these cytotoxic effects are circumvented.

In support of such ideas about the balance between influx and efflux, the plasma membrane, Ca^{2+} efflux pump ACA9 appears critically important for normal pollen tube function (Schiott et al. 2004). Thus, amongst the 14 Ca^{2+} pumps identified in the *Arabidopsis* genome, ACA9 belongs to a ten-member subfamily of autoinhibited Ca^{2+} ATPases thought to be regulated by Ca^{2+}/calmodulin (CaM; Baxter et al. 2003). ACA9 is principally pollen expressed and mutants in this gene

show reduced fertility through male sterility (Schiott et al. 2004). Analysis of the distribution of fertilization within siliques strongly suggests that the *aca9* pollen tubes fail to elongate successfully within the carpel and so fail to fertilize ovules further into the flower (Schiott et al. 2004). Thus, disruption of the activity of a pollen-expressed Ca^{2+} efflux pump appears to have profound effects on pollen tube function, consistent with a central role of Ca^{2+} efflux on the tip growth process. ACA9 is likely to be just one of a family of Ca^{2+} pumps whose integrated activities are required to curtail the extent of the Ca^{2+} gradient driving growth in tip-growing cells.

The other side of the regulatory systems controlling the Ca^{2+} gradient is the Ca^{2+} influx channel(s) and the mechanisms leading to localized gating at the site of growth. The means behind spatially restricting channel activation are likely to be the critical elements imposing localization to the Ca^{2+} gradient and so to the growth machinery. Data from pollen growth suggests a cyclic nucleotide-gated channel (CNGC) is one essential component of polarized tip growth. The CNGCs are non-specific cation channels found in both plants and animals. They are thought to be regulated by changes in the cytosolic levels of cyclic nucleotides such as cGMP (Talke et al. 2003). There are 20 predicted CNGCs in the *Arabidopsis* genome and of these a knockout in the pollen-expressed CNGC18 causes male sterility through pollen tube growth defects where elongating pollen tubes become thin, kinked, and unable to traverse the transmitting tract of the style (Frietsch et al. 2007). CNGC18 shows localization to the growing tip, providing an extremely strong candidate for a Ca^{2+} conductance driving the Ca^{2+} gradient associated with tip growth. Whether such tip-focused localization of the channel is also associated with localized gating of the channel through, for example, a gradient of cyclic nucleotide levels has yet to be defined. However, consistent with these ideas, experimentally induced alterations in cytosolic cyclic nucleotide levels do appear to trigger growth-altering changes in the tip-focused Ca^{2+} signal (Moutinho et al. 2001).

A second candidate for a channel driving the tip-focused Ca^{2+} gradient comes from electrophysiological analysis of channel activities found in protoplasts isolated from the tips of root hairs. If these protoplasts retain the characteristics of the membrane at the growing tip, then they should be enriched in the channels driving the Ca^{2+} gradient. Indeed, such analysis showed a hypepolarization-activated channel to be enriched in the apex of the root hair (Foreman et al. 2003; Miedema et al. 2008; Very and Davies 2000). This conductance was also gated by elevated reactive oxygen species (ROS). The significance of this sensitivity to ROS lies in the characterization of one mutant defective in root hair tip growth as being caused by lesions in a ROS-generating enzyme. Thus, the *r*oot *h*air *d*efective *2* mutant (*rhd2*) of *Arabidopsis* disrupts root hair formation by failing to initiate root hair tip growth once the site of the future root hair has been formed (Foreman et al. 2003). Tip growth and initiation are genetically separable processes with initiation being characterized by the local bulging of the root epidermal cell wall that then transitions to the process of tip growth (reviewed in Bibikova and Gilroy 2009). The bulging process is characterized by events such as accumulation of

expansins and endotransglycosylases (Baluska et al. 2000; Vissenberg et al. 2001) and localization of G-proteins (Carol et al. 2005; Jones et al. 2007; Jones et al. 2002; Molendijk et al. 2001) that predict the site of wall deformation and by localized acidification of the wall (Bibikova et al. 1998) that may promote acid growth-mediated bulging. However, unlike the process of tip growth, no Ca^{2+} gradient has been detected that either predicts the site of root hair formation, or that accompanies this initiation-related wall bulging process (Wymer et al. 1997). The *rhd2* mutant forms initiation bulges on the sides of epidermal cells but these fail to enter tip growth indicating a role in the tip growth phase of root hair formation. The relevant mutated gene was eventually identified as the *r*espiratory *b*urst *o*xidase *h*omolog C of *Arabidopsis* (AtRBOHC), a homolog of the mammalian $gp91^{phox}$ catalytic subunit of the mammalian NADPH oxidase responsible for ROS production during the phagocytic oxidative burst (Foreman et al. 2003). In plants, these enzymes oxidize cytosolic NADPH to generate superoxide to the apoplast where the superoxide radical is rapidly dismutated to H_2O_2 (Fig. 1a). The apoplastic H_2O_2 is thought to leak back into the cell, possibly through channels such as aquaporins (Bienert et al. 2007; Dynowski et al. 2008), where it can act as a cytsolic regulator. Thus, the identification of an NADPH oxidase as a critical element of tip growth implies a role for ROS in regulating this process. Treating *rhd2* plants with H_2O_2 facilitates enlargement of the initiated root hairs but they do not recover the polarized nature of their growth, leading to spherical bulges on the side of the root (Foreman et al. 2003). This observation suggests that the H_2O_2 can support the "tip growth" process in the *rhd2* background but simply adding it to the plant does not recreate the highly localized nature of cell expansion associated with the polar growth process, i.e., the spatial control on the process has been lost. AtRBOHC expression is limited to the epidermal cells of the root just at the point where root hair formation is starting. Indeed, the protein accumulates at the initiation points of root hairs (Takeda et al. 2008), yet the lack of a phenotype in the root hair initiation process implies that AtRBOHC is either inactive or that other ROS producing enzymes mask the effect of the *rhd2* lesion in the initiation process. The absence of a detectable Ca^{2+} gradient during the initiation process in wild-type plants might also explain the lack of a phenotype for *rhd2* during initiation if this NADPH oxidase is acting through regulation of Ca^{2+} channel activities. Upon the transition to tip growth, this protein localizes to the apex of the elongating hair, suggesting it could be responsible for a localized gradient in ROS focused to the growing tip and so gating the ROS-sensitive channel.

A tip-focused gradient in ROS has been reported in pollen tubes (Potocky et al. 2007) and tip growing algal rhizoids, which also show a tip-focused Ca^{2+} gradient thought to drive growth (Coelho et al. 2008; Coelho et al. 2002). Pollen tube growth is also sensitive to inhibitors that target flavin containing enzymes including the NADPH oxidases (Potocky et al. 2007), providing further evidence that a conserved ROS-related mechanism may be at play in the regulation of diverse tip growing cells.

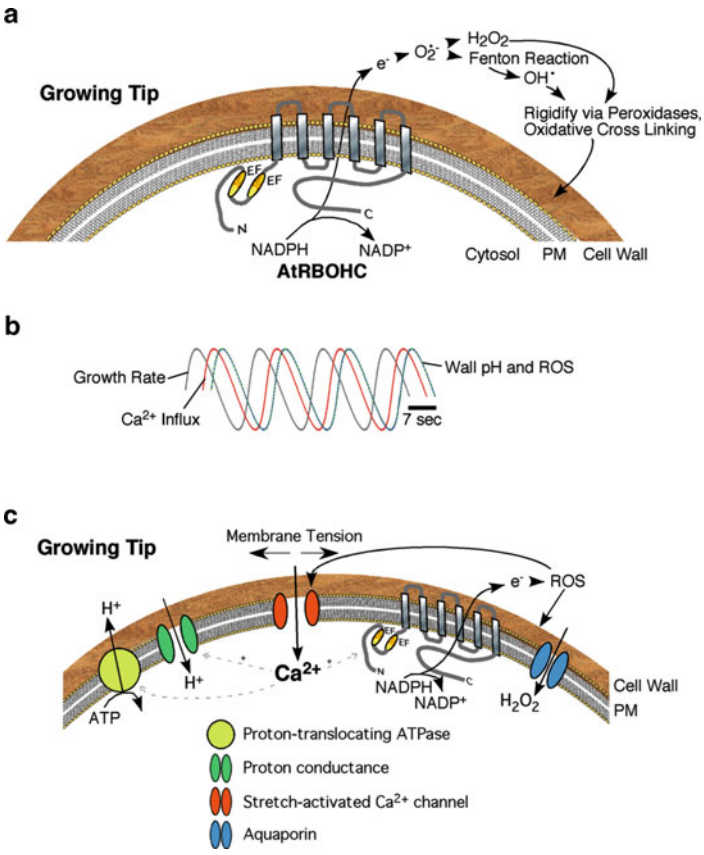

Fig. 1 *Tip growing plant cells: ROS production, Ca^{2+} dynamics in relation to growth and wall pH, and a model of activities at the growing tip.* (**a**) The structure and enzymatic activity of AtRBOHC during tip growth. AtRBOHC is a NADPH oxidase which contributes to the production of ROS in the apoplast. The ROS made then go on to affect cell wall strength and downstream signaling in the cytosol. (**b**) Kinetics of growth rate, cytoplasmic Ca^{2+} concentration, and wall pH/ROS during tip growth. Peak Ca^{2+} follows maximal growth by a few seconds, followed by a peak in cell wall pH and ROS. (**c**) Model of the key players regulating tip growth. Growth causes an increase in the membrane tension which causes stretch-activated Ca^{2+} channels to open, resulting in a rise in cytoplasmic Ca^{2+}. The elevated Ca^{2+} affects the activity of proton conductances, possibly ATPases and proton influx carriers, leading to increased wall pH that helps to stabilize the newly formed wall at the tip. Increased cytosolic Ca^{2+} also affects the activity of the NADPH oxidase and so alters ROS in the apoplast; this ROS can also rigidify the new cell wall in addition to entering the cytosol via aquaporins to facilitate ROS-dependent signaling in the cytosol

3 Stretch-Activated Signals and Growth

In addition to this ROS-based system, there is accumulating evidence from both pollen tubes and root hairs that the stretch inherent in turgor-based elongation may be a significant player in gating mechanosensitive Ca^{2+} permeable channels that can drive growth. One key observation has come from analysis of the temporal series of events related to cell expansion in these apically growing cells. Thus, many tip growing cells, including pollen tubes and root hairs, show oscillatory expansion with periods of rapid elongation interspersed with slower periods of growth. Likewise, the tip-focused gradient in Ca^{2+} has been characterized as oscillating in magnitude with approximately the same period as growth (e.g., Messerli et al. 2000; Monshausen et al. 2008a; Pierson et al. 1996). When these oscillations were correlated, the maximum of the Ca^{2+} gradient was actually seen to trail the increase in growth by several seconds (Messerli et al. 2000; Monshausen et al. 2008a; Fig. 1b). This timing was consistent in both root hairs and pollen tubes despite an approximately tenfold difference in their absolute growth rates, hinting at a possibly conserved mechanistic component of the oscillator driving these phenomena. When ROS changes were also added to a temporal and spatial mapping of events, extracellular ROS levels were seen to oscillate at the tip of the growing cell and, in root hairs, were characterized to follow the Ca^{2+} increase by a further 5 s. These observations suggest a model where the membrane stretching induced at the expanding apex of the cell by the processes of tip growth might trigger stretch-activated Ca^{2+} permeable channels. Ca^{2+} influx would then follow growth and possibly trigger ROS production (Fig. 1c). Consistent with this model, a stretch-activated Ca^{2+} channel has been identified in tip growing pollen tubes (Messerli and Robinson 2007). The link between Ca^{2+} increase and ROS production is strengthened by the observations that increasing the level of Ca^{2+} in the medium can trigger ROS production in pollen tubes (Potocky et al. 2007) and experimentally increasing cytosolic Ca^{2+} by using Ca^{2+} ionophore treatment can trigger AtRBOHC-dependent ROS production to the apolast in root hairs (Monshausen et al. 2007). Indeed, AtRBOHC contains EF-hand motifs that are classic hallmarks of a Ca^{2+}-sensitive protein (Fig. 1a). These domains are essential for the role of this enzyme in supporting tip growth in root hairs because a mutated version of AtRBOHC where the Ca^{2+} binding activity of the EF hands has been disabled was unable to complement the *rhd*2 phenotype, whereas wild-type AtRBOHC was (Takeda et al. 2008). In addition to a direct role for Ca^{2+}, phosphorylation by Ca^{2+}-dependent protein kinases also seems an important element in the regulation of these NADPH oxidases. Thus, eliminating the target site for phosphorylation on AtRBOHC severely reduced its capacity to produce ROS (Takeda et al. 2008).

How then does the Ca^{2+}-dependent activation of NADPH oxidase and production of extracellular ROS fit with the cytosolic ROS-gated Ca^{2+} channel thought to play a role in regulating tip growth? There appears to be a complex feedback

system in play where growth may trigger a stretch-activated Ca^{2+} conductance triggering ROS production to the wall. This ROS then transits to the cytosol where it may reinforce the Ca^{2+} signal through its action on the ROS-sensitive channel (Fig. 1c).

4 G-Proteins and Tip Growth

Monomeric G-proteins (Rops, Racs) also appear to be integral components of this regulatory loop, although their precise relationship to the Ca^{2+} gradient remains to be fully explored. Thus, the Rops are known to be localized to the apex of tip-growing cells and have been closely linked to the maintenance of apical growth (Lee and Yang 2008). Constitutively active mutants of Rops 2, 4, and 6 all lead to increased root hair length and uncontrolled expansion leading to tip bulging. In addition, overexpression of ROP2 leads to elevated ROS production in root hairs that is dependent on AtRBOHC (Jones et al. 2007). One clue to a possible interaction with Ca^{2+} is that the monomeric G-protein OsRac1 directly binds to rice NADPH oxidase in vivo in a Ca^{2+}-dependent manner (Wong et al. 2007). There also appears a key role for the GTP dissociation inhibitors (RhoGDIs) and GTPase activator proteins (RhoGAPs) of these GTPases in this regulatory system. The RhoGDIs maintain retention of GDP bound to the G-protein holding it in its "off" state, whereas GAPS promote GTPase action, turning over bound GTP and so terminating G-protein-dependent signaling events. One mechanism for RhoGDIs and GAPS to localize growth to the tip may be through their roles in restricting the action of ROPs to the very apex of the cell. The supercentipede mutant in *Arabidopsis* has a lesion in a RhoGDI and forms multiple growing tips from the cell that would normally form a single root hair (Carol et al. 2005; Takeda et al. 2008). This mutant appears to mislocalize ROS production in these cells through mislocalization of the G-proteins and the NADPH oxidase (Takeda et al. 2008). Normal localization of these proteins may well rely upon the action of the microfilament cytoskeleton. In an actin2 knockout, AtRBOHC was mislocalized and root hair tip growth disrupted; similarly, pharmcological disruption of microfilaments, but not microtubules, led to misplacement of the NADPH oxidase (Takeda et al. 2008). The actin cytoskeleton is itself sensitive to regulation by ROPs and a range of Ca^{2+}-dependent regulatory proteins (Bibikova and Gilroy 2009). Two direct targets of the ROPs, RIC3 and RIC4 (ROP Interacting Clone 3 and 4) are thought to regulate Ca^{2+} signaling and actin dynamics, respectively (Gu et al. 2005), providing a potential link between the ROP-dependent and Ca^{2+}-regulated systems. These observations suggest conserved features of the growth machinery between tip growing plant cells of gradients in the activity of G-proteins, tip-focused Ca^{2+}, and ROS-related events despite the very different growth rates and functions of each kind of apically growing cell.

5 Apoplastic ROS and Changes in pH

Such a complex regulatory system controlling apical growth is perhaps not surprising as tip growing cells are likely operating near the limits of controlled expansion. They must carefully balance weakening of the tip cell wall sufficiently to allow turgor to drive elongation with too much wall relaxation and runaway expansion leading to bursting. Therefore, one might expect a carefully monitored and modulated series of events at the tip to maintain this dance between growth and potential death. In keeping with such ideas of a highly integrated, likely redundant, regulatory network, the apoplastic ROS produced under these circumstances appears to play important roles other than as a simple source for the intracellular ROS signal. Thus, depending on growth conditions, up to 100% of *rhd2* root hairs can be seen to burst at the transition to tip growth (Macpherson et al. 2008; Monshausen et al. 2007), implying a lesion in wall integrity as turgor-driven tip growth begins to progress. Extracellular ROS have been proposed to play important roles in wall structure such as through cross-linking of wall polymers and so contributing to the irreversible rigidification of the wall (Muller et al. 2009; Pedreira et al. 2004; Potikha et al. 1999; Schopfer 2001). Thus, a lesion in the NADPH oxidase generating these extracellular ROS at the expanding tip might be expected to have the observed weakening effect on the apical wall. Indeed, visualization of extracellular ROS (superoxide) production during growth in root hairs showed an oscillatory component that lagged behind the oscillations in growth, whereas a steady production of ROS occurred to the flanks of the cells (Monshausen et al. 2007). Scavenging of ROS led to tip bursting, whereas addition of exogenous ROS caused inhibition of elongation, consistent with a role for this extracellular ROS in rigidifying the apical wall (Monshausen et al. 2007).

In addition to fluctuations in ROS production, oscillations in apoplastic pH are also seen to occur in phase with oscillations in tip growth in both pollen tubes and root hairs (Monshausen et al. 2007; Robinson and Messerli 2002). These elevations in pH occur concurrently with the increases in apoplastic ROS, trailing the Ca^{2+} increase by several seconds. As with the ROS increase, these pH elevations can be triggered by artificially increasing cytosolic Ca^{2+} levels (Monshausen et al. 2007), consistent with them being caused by the same Ca^{2+} increase that activates AtRBOHC to produce apoplastic ROS. Increased wall pH is thought to slow growth by, for example, reducing the activity of proteins such as the expansins that mediate acid growth (Sampedro and Cosgrove 2005). Thus, the oscillations in elevated pH seen after a pulse of growth may be playing a similar role to the burst of wall ROS that occurs at the same time in rigidifying the wall after a period of rapid expansion. This slowing in growth would allow wall and membrane synthesis to catch up with expansion and so reinforce the wall to prevent bursting (Fig. 1c). Consistent with this model, artificially elevating apoplastic pH inhibits root hair elongation, whereas reducing it promotes bursting (Monshausen et al. 2007). Interestingly, elevating pH can also rescue the aborted tip growth/bursting phenotype of the *rhd2* mutant, suggesting that the apoplastic pH and ROS systems are playing equivalent

roles and can to some degree compensate for the lack of each other. These "rescued," tip growing *rhd2* root hairs show a normal tip-focused Ca^{2+} gradient (Monshausen et al. 2007). This observation suggests that the ROS-dependent gating of apical Ca^{2+} channels via the action of NADPH oxidase C is not a required component of the tip growth process, with either another ROS-producing enzyme, or other channel system such as the stretch-activated conductances, compensating for the loss of AtRBOHC-dependent events. Again, this may not be too surprising if the tip growth machinery has multiple redundant regulatory mechanisms to ensure the correct balance between wall rigidity, growth, and turgor pressure.

It is also important to note that lesions in the NADPH oxidases not only disrupt tip growth but also have more widespread effects on organ growth and development. *rhd2* plants show root elongation that is retarded by approximately 20% (Foreman et al. 2003; Renew et al. 2005) and lesions in another two of the *Arabidopsis* NADPH oxidases, AtRBOH D and F also show reduced growth (Torres and Dangl 2005). Thus, there may well be key parallels between the series of Ca^{2+}-, ROS-, and pH-related events defined from tip growth and growth responses seen elsewhere in the plant. This idea is supported by observations on mechanical sensing throughout the plant as described below.

6 Calcium and Mechanical Signal Transduction

Mechanical stimulation of the plant either by point contact, shaking, wind or by bending elicits an increase in cytosolic Ca^{2+} levels with an initial rapid, transient elevation of a few seconds that, depending on stimulus type, can be followed by a slower more sustained increase lasting tens of seconds (reviewed in Monshausen et al. 2008b). Initial experiments indicated that these changes could not be prevented by putative blockers of plasma membrane channels, such as La^{3+}, implying that they were being generated by release from internal Ca^{2+} stores. However, we now know that high extracellular Ca^{2+} can effectively compete with La^{3+} and so suppress its effects on Ca^{2+} changes (Monshausen et al. 2009; Monshausen et al. 2008a). When extracellular Ca^{2+} is lowered to below 1 mM, La^{3+} is effective at blocking mechanically induced Ca^{2+} increases (Monshausen et al. 2009), suggesting that at least the initial Ca^{2+} influx in response to touch is dependent on influx across the plasma membrane. Whether the second phase of the response requires a "priming" influx across the plasma membrane triggering calcium-induced calcium release from internal stores remains to be determined.

Imaging of these mechanically induced Ca^{2+} responses in e.g. the root undergoing mechanical stimulation, has shown that there are in fact distinct patterns to the Ca^{2+} increase dependent on the kind of stimulation. Point contact that may mimic, for example, the initial events of fungal invasion (Hardham et al. 2008), elicits a localized Ca^{2+} increase that spreads from the point of contact on the cell (Monshausen et al. 2009). In contrast, in roots undergoing bending, such as occurs when they grow into a barrier in the soil, a biphasic Ca^{2+} increase is elicited

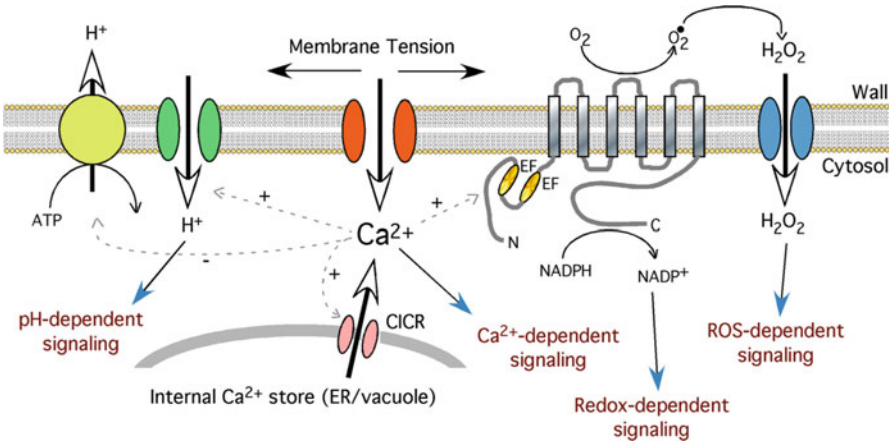

Fig. 2 *Model for mechanical-related signaling*. Mechanical stimulation leads to membrane tension that opens a Ca^{2+} permeable stretch-activated channel. Ca^{2+} influx into the cytosol then may activate a Ca^{2+}-induced Ca^{2+}-release channel (CICR) causing a second phase of Ca^{2+} increase in the cytosol. The elevated Ca^{2+} levels lead to inhibition of H^+-efflux from the cell and activation of H^+-influx. Simultaneously, increased Ca^{2+} causes activation of NADPH oxidase leading to increased superoxide levels in the apoplast. These are rapidly dismutated to H_2O_2 that then enters the cell. The alterations in Ca^{2+}, H^+, ROS, and redox balance could all lead to cytosolic signaling events. Note the shared components and similarities in signaling cascades to tip growth shown in Fig. 1

but only in those cells under tension, i.e., in the convex side of the curve in the root (Monshausen et al. 2009; Richter et al. 2009). Cells undergoing compression show no equivalent Ca^{2+} increase. Such a spatial distribution of Ca^{2+} elevation is consistent with a role for the activation of a stretch-sensitive Ca^{2+} permeable channel, similar to the predictions for the channel at the apex of tip growing cells described above. Stretch-stimulated cells also show a burst of apoplastic ROS production and pH elevation (Gus-Mayer et al. 1998; Monshausen et al. 2009; Yahraus et al. 1995) that is dependent on the increase in cytosolic Ca^{2+} (Monshausen et al. 2009), again bearing striking parallels to the changes seen during tip growth. It seems likely that pH, ROS, and cytosolic Ca^{2+} represent a conserved, functional cassette that can translate membrane tension into a ROS and pH response (Fig. 2). This system may have evolved to respond to the membrane stresses inherent in turgor-driven expansion to help coordinate growth with wall characteristics but similar changes can be seen in tip growth and are even hinted at in a range of abiotic and biotic stress responses (e.g., Torres and Dangl 2005). Such changes in wall properties might help to maintain cell integrity despite the competing need to loosen the cell wall to allow growth and reinforce the wall under strain but now may have been co-opted to reinforce the wall or adapt growth to deal with a range of stresses the plant experiences.

7 Mechanosensory Channels

Parallels in Ca^{2+} signaling from tip growth to mechanical responses exist throughout the plant, however, whether they use similar Ca^{2+} channels is less clear. As ROS appear intimately linked to mechanosignaling, testing for a mechanosensory role for the hyperpolarization/ROS-gated channel thought to drive tip growth is a pressing goal. Although the CNGCs have been shown to have roles in a range of responses from pollen tube growth (Frietsch et al. 2007) to defense signaling (Ali et al. 2007) there is little current evidence for their role in mechanosensation and the top candidates at present for mechanosensory channels are unrelated to the CNGCs, i.e. the MSL and MCA proteins.

The MSL (MscS-like) genes are plant homologs of the mechanosensitive channels (MscS) from bacteria (reviewed in Monshausen et al. 2008b). The rice genome contains six MSLs, whereas *Arabidopsis* has ten. AtMSL1 likely plays a role in mitochondria and AtMSL2 and 3 participate in plastid division (Haswell and Meyerowitz 2006). The physiological function of the other members of this gene family is less clear. MSL9 and 10 occur in the plasma membrane of root cells and appear involved in mechanosensitive gating of a Cl^- conductance (Haswell et al. 2008). However, a quintuple knockout of all root-expressed MSLs (*msl4*/*5*/*6*/*9*/*10*) has no overt phenotype and responds as wild type to osmotic and mechanical stresses (Haswell et al. 2008).

Another strong candidate for a component of a mechanosesitive channel is MCA1. This protein was found in a screen to complement the *mid1* knockout in yeast with *Arabidopsis* cDNAs (Nakagawa et al. 2007). MID1 is a component of a yeast stretch-activated channel. AtMCA1 has a single paralog, MCA2, that also complements the yeast MID1 Ca^{2+} uptake phenotype (Yamanaka et al. 2010), but neither shares extensive structural similarity to MID1 (MCA1 is only 10% identical and 41% similar to MID1). The *mca1* (but not *mca2*) mutant shows a reduced ability to penetrate hard agar, and in MCA1 overexpressing lines touch responsive genes such as TCH3 (Cml11) are upregulated, suggesting a possible relationship to mechanical sensing/response. *mca2* knockout plants showed reduced Ca^{2+} uptake by roots and heterologous expression of MCA1 in mammalian CHO cells created a novel stretch-induced Ca^{2+} current (Nakagawa et al. 2007; Yamanaka et al. 2010). Thus, the links between Ca^{2+} and MCA1 and 2 are strong but whether these proteins form a stretch-activated Ca^{2+} channel remains to be defined at the molecular and electrophysiological levels.

8 Encoding Information in the Calcium Signal

One question arising from the above discussion of growth and mechanically coupled changes in Ca^{2+}, is could such stimulus-specific Ca^{2+} signals be processed to unique outcomes in the regulation of growth? One possibility is that Ca^{2+}

elevations simply act as threshold-based switches, with their complex dynamics simply reflecting features of the Ca^{2+} homeostatic system and not carrying significant information (Scrase-Field and Knight 2003). Alternatively, unique Ca^{2+} signatures monitored at the whole plant level could actually reflect cell-type specific signals, with particular tissues or cells eliciting distinct spatial and/or temporal changes in Ca^{2+} that then lead to cell-type specific response. For example, when monitored at a whole plant level using the Ca^{2+}-sensitive luminescent photoprotein aequorin ubiquitously expressed in all cell types, cold stress leads to a characteristic temporal Ca^{2+} signature (Fig. 3a, 35S data). However, when the same Ca^{2+} reporter was monitored using transactivation lines to drive cell-type specific expression of the sensor, distinct differences in this signature were seen between cell types (Kiegle et al. 2000). Indeed, such cell type specificity of

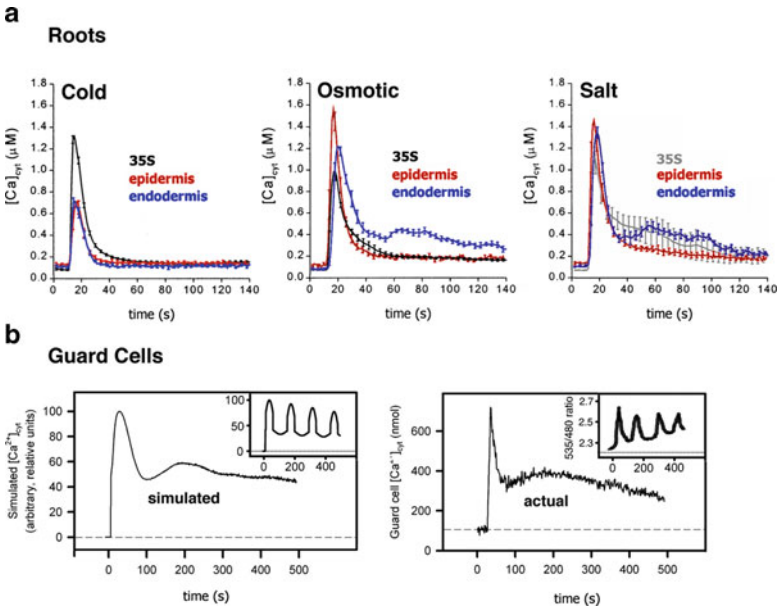

Fig. 3 *Comparison of the calcium response of entire organs compared to individual cell types, and of a population of cells compared to a single cell*. (**a**) Cytoplasmic calcium changes over time as reported by aequorin in an *Arabidopsis* root (35S::aequorin) or in specific cell types of the root in response to either cold, osmotic, or salt stress. Note the differences in kinetics that are revealed when observing the calcium response in only the epidermis (*red*) or endodermis (*blue*) instead of the entire organ (*grey*). Redrawn from Kiegle et al. 2000. (**b**) Simulated and actual *Arabidopsis* guard cell cytoplasmic calcium dynamics in response to cold shock in either a population or a single cell. The simulation (*left panel*) is an average of 5,000 individual oscillating cells (*inset*) showing an initial synchronous peak followed by a *broader peak* that represents an amalgamation of asynchronous oscillations. Actual aequorin measurements of the calcium signature in a guard cell population responding to cold shock (*right panel*) match the simulation, and the more sensitive ratiometic fluorescent calcium indicator yellow cameleon reveals oscillations in a single guard cell after cold shock (*inset*). Inset axis labels are the same as those on the outset graph, unless otherwise marked. Redrawn from Dodd et al. (2006) and Allen et al. (2000)

response was seen to a range of stimuli (Fig. 3a). Work in animal cells shows that even in the same cell, different magnitudes of Ca^{2+} elevation can lead to different suites of response elements being triggered (Dolmetsch et al. 1997). Thus, the same stimulus could be driving very different responses in different cell types and averaging the Ca^{2+} change over the whole organism may provide a misleading idea of the kinetics of the response at the cellular level. This point was highlighted by modeling the kinetics of Ca^{2+} change expected from stomatal guard cells responding to low temperature. When assayed at the single cell level using imaging of a Ca^{2+}-sensitive GFP (Cameleon YC2.1), oscillations were detected in cold stressed stomatal guard cells (Fig. 3b, right panel insert; Allen et al. 2000), whereas at the whole plant level using aequorin, which averaged response of many stomata, a spike and then shoulder of elevated Ca^{2+} was recorded (Fig. 3b, right panel; Dodd et al. 2006). Modeling showed that the initial spike in the whole plant aequorin measurements likely reflected a synchronous response from the cells, whereas the shoulder was produced by averaging of the subsequent oscillations exhibited at the single cell level slowly falling out of phase with each other (Fig. 3b, left panel; Dodd et al. 2006).

Alternatively, the same cell type could be generating unique Ca^{2+} signatures to different stimuli. In this case there would need to be a mechanism to decode the spatial and temporal characteristics of these changes to trigger unique outcomes. Thus, for spatial encoding/decoding, the localization of Ca^{2+}-regulated proteins and Ca^{2+} change within the cell could have profound effects on what downstream changes a spatially localized change in Ca^{2+} could elicit. An obvious example would be Ca^{2+} changes elicited in the nucleus where no equivalent simultaneous cytosolic change occurs, as observed, for example, in the Ca^{2+} spiking localized to the nucleus of root hair cells responding to NOD factors (Ehrhardt et al. 1996). NOD factors are signaling compounds produced by symbiotic bacteria such as rhizobia as part of the nodulation signaling pathway of nitrogen fixing symbiosis (Stacey et al. 2006). In this case, only nuclearly localized Ca^{2+}-dependent response factors would be triggered to elicit nuclear response pathways, despite a host of Ca^{2+}-dependent factors also being present in the cytosol. There is evidence that such spatially localized encoding of response can be extended to even subtler localizations. Perhaps one of the best examples is seen in mammalian neurons where Ca^{2+} influx through L-type channels in the plasma membrane triggers activation of the CREB transcription factor. Even though Ca^{2+} entry through non-L-type channels can elicit a Ca^{2+} increase similar to that of the L-type channel it fails to trigger the signaling cascade leading to CREB activation (Dolmetsch et al. 2001; West et al. 2001). This specificity appears to reside in a CaM molecule bound via an "IQ" motif to the inner surface of the L-type channel. Activation of this specific CaM is needed to activate the signaling cascade to CREB and only Ca^{2+} passing through the L-type channel has access to this protein. There are numerous examples of spatially localized Ca^{2+} changes in response to myriad signals in plants, suggesting this kind of "hard wired" signal processing could be widespread.

In addition to the spatial component of signal processing, plants also are capable of generating complex temporal changes in Ca^{2+} levels that could also carry

information to trigger unique responses. In stomatal guard cells many stimuli elicit Ca^{2+} changes linked to stomatal closure (Kim et al. 2010). These Ca^{2+} signals are often seen to oscillate and by experimentally manipulating cytosolic Ca^{2+} it has been possible to impose Ca^{2+} transients with a range of frequencies. These experiments have revealed two mechanisms behind Ca^{2+}-dependent signals: short-term Ca^{2+}-reactive closure and long-term Ca^{2+}-programmed closure. Ca^{2+}-reactive closure is a threshold phenomenon where Ca^{2+} increases to above a critical level and induces response. The temporal character of the Ca^{2+} spiking in this case is not relevant. However, Ca^{2+}-programmed closure is dependent on the "signature" of the Ca^{2+} change, being sensitive to the frequency and duration of the Ca^{2+} spiking. These are genetically separable elements of Ca^{2+} signal processing; for example, a double mutant in the downstream Ca^{2+}-regulated protein kinases CPK3 and CPK6 show impaired Ca^{2+}-reactive closure but unaltered Ca^{2+}-programmed closure. Conversely overexpression of the glutamate receptor homolog AtGLR3.1, disrupts Ca^{2+} programmed closure but not the rapid Ca^{2+}-reactive closure (Cho et al. 2009).

Thus, it appears that, at least in the case of the guard cell, there should be information about the stimulus encoded in the temporal characteristics of the Ca^{2+} signal. How then might a cell take such information and decode it into a biochemical change in a signaling pathway? In animal cells, a similar Ca^{2+}-dependent frequency-encoding element has been observed and a possible biochemically based decoding system has been defined in the characteristics of CaM-dependent kinase II. Here the kinetics of association and dissociation of Ca^{2+} with CaM and of Ca^{2+}-activated CaM with the kinase leads to a bistable switch where only when Ca^{2+} transients are of sufficient magnitude and of sufficient frequency is the kinase activity switched on (De Koninck and Schulman 1998). Different subunit isoforms show different frequency responses allowing the cell to tune to the signal it wishes to decode. Although this mechanism was initially defined in animals, it may well play a similar role in NOD factor signaling where the Ca^{2+}/CaM-dependent kinase (DMI3 or CCaMK) is known to be an important downstream component taking the Ca^{2+} signal elicited by NOD factor perception and then translating this to transcriptional activation in the nucleus (Ane et al. 2004; Gleason et al. 2006; Levy et al. 2004; Mitra et al. 2004a, b; Tirichine et al. 2006). CCaMKs have an autoinhibitory domain that negatively regulates the kinase (Patil et al. 1995) and deletion of this domain in DMI3 leads to constitutive nodulation signaling (Gleason et al. 2006; Tirichine et al. 2006), reinforcing the idea that Ca^{2+}-dependent activation of this kinase is critical to NOD factor signaling. Interestingly, the evidence suggests that this system is able to count the number of Ca^{2+} spikes rather than utilize information in the frequency domain. Thus, when monitoring induction of the Nod factor responsive ENOD11 promoter, it was observed that a threshold of 36 transients was required to trigger response and that the frequency component of the response was not as critical as absolute number of transients (Miwa et al. 2006).

9 Role of CaM and CDPKs in Growth

There is evidence that these kinds of Ca^{2+}-dependent response components might also play similar roles in translating Ca^{2+} signals to the modulation of growth. For example, proteins such as CaM and calcium-dependent protein kinases (CPKs or CDPKs) are thought to play important roles during tip growth. Thus, CaM exhibits a tip-focused gradient in pollen and utilizing a fluorescent indicator of CaM activity, Rato and colleagues found this apically localized CaM pulsed in its state of activation with a period of 40–80 s (Rato et al. 2004). Intracellular activation of a CaM inhibitory peptide caused reorientation of growth, suggesting CaM may play a role in the tip growth guidance system. The pollen-specific *Arabidopsis* protein NPG1 (No Pollen Germination 1) appears to be a target for three CaM isoforms (CaM2, 4, and 6) and genetic, histological, and pollen germination analyses suggests it plays an important role in pollen germination (Golovkin and Reddy 2003).

In addition, several studies have characterized an important role for CDPKs in the tip growth of pollen tubes and root hairs (reviewed in Kudla et al. 2010). It appears that the spatio-temporal patterns of expression and accumulation of CDPKs are important in regulating tip growth. For example, using a fluorescent probe for CDPK activity, Moutinho and colleagues reported CDPK activity to show a tip-focused gradient and increases in activation were seen to accompany redirection of pollen tube growth, suggesting a role in the apical growth machinery (Moutinho et al. 1998). In maize, *ZmCDPK* showed pollen specific expression (Estruch et al. 1994) and using the pharmacological CaM and CDPK inhibitors calmidazolium and W-7, and *ZmCDPK* mRNA antisense oligonucleotides, In vitro pollen germination and tube growth were shown to be dependent on CaM/ZmCDPK action. In addition, Yoon and colleagues showed that *Petunia inflata* CDPK1 (PiCDPK1) and PiCDPK2 play distinct roles in pollen tube growth polarity (Yoon et al. 2006). Transient expression of PiCDPK1 and PiCDPK2 showed that these proteins localize to different subcellular compartments: the plasma membrane for PiCDPK1 and unknown internal compartment for PiCDPK2. Expression of a catalytically inactive and a calcium-independent (constitutively active) version of these enzymes caused disruption of pollen tube elongation and of the tip-focused Ca^{2+} gradient showing that the function of the PiCDPKs is associated with the regulation of pollen tube growth, possibly involving maintenance of Ca^{2+} homeostasis. A requirement for the function of CDPKs has also been shown in the model plant *Arabidopsis* (Myers et al. 2009). Thus, both AtCPK17 and AtCPK34 are targeted to the plasma membrane during pollen tube germination and tube growth. Homozygous double knockout mutants of AtCPK17 and AtCPK34 showed a near-sterile phenotype which was rescued by expression of AtCPK34, suggesting that the functions of the CPK17 and CPK34 proteins are redundant and essential to pollen tube growth.

Taken together, these observations suggest that the function of the CDPKs is important for pollen tube growth, most likely by decoding the Ca^{2+} gradient generated during pollen tube growth. This role appears conserved in root hair

elongation as well. A RNA interference line of *Medicago truncatula* directed against MtCDPK1 showed both a reduced number of root hairs and root cell length, resulting in reduction of rhizobial and mycorrhizal symbiotic colonization (Ivashuta et al. 2005). According to microarray analysis of these plants, silencing of *MtCDPK1* led to altered expression of genes relating to cell wall formation and defense. These findings are consistent with the idea that the function of MtCDPK1 is to modulate cell expansion or cell wall synthesis of Medicago during plant and microbe symbiotic interactions.

10 Conclusions and Perspectives

The above discussion highlights a role for Ca^{2+} as a ubiquitous regulator of growth. There also appears a close connection between Ca^{2+}, ROS, and proton fluxes that may be linked in a conserved response cassette. For tip growth we are beginning to amass a series of molecularly identified components of this response system from identified Ca^{2+} channels generating the tip-focused Ca^{2+} gradient sustaining growth, to elements of the monomeric G-protein complex, to response components such as the CDPKs that integrate Ca^{2+} change to cellular response. There are strong parallels to the mechanical signaling system elsewhere in the plant but here we are currently lacking equivalent molecular resolution of the signaling components. Thus, identification of mechanically responsive Ca^{2+} permeable channels and response components such as potentially afforded by CaM or the CPKs is a pressing need for the field. Interestingly, some of the earliest touch-inducible transcripts identified, the TCH genes, encode for CaM or CaM-like proteins (McCormack et al. 2005), suggesting touch may be able to remodel its own Ca^{2+} response network. Indeed, in general these classes of Ca^{2+}-responsive proteins are known to play roles in many different processes ranging from the regulation of trichome development (through modulation of kinesin interacting Ca^{2+}-binding protein by cml42; Dobney et al. 2009), to transcriptional regulation during defense signaling (CaM-binding transcription factor 3; Du et al. 2009; Galon et al. 2008). One unexpected recent finding was that *Arabidopsis* CaM7 can even function directly as a transcription factor specifically binding to the *cis*-acting Z-/G-Box (ATACGTGT/CACGTA) light-responsive element in promoters of light-inducible genes, suggesting that *AtCaM7* may regulate expression of genes in response to light signals (Kushwaha et al. 2008). As such functions continue to be discovered and the spectrum of Ca^{2+} binding proteins in plants (such as the CaMs, CaM-like, CDPKS, CBL/CIPKs, CaMTAs; Dodd et al. 2010; Kudla et al. 2010) become better defined we can anticipate the molecular mechanisms behind the pervasive role of Ca^{2+} in plant regulation to become more clear. The possibility of a Ca^{2+} signal being transduced through many simultaneous Ca^{2+} response networks, each tuned to a specific component of the Ca^{2+} signal, holds the potential to explain in part how plants

can integrate such a wide spectrum of stimuli to establish the plastic development that is a hallmark of plant responses to an ever-changing world.

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Calcium Signals in the Control of Stomatal Movements

Alex A.R. Webb and Fiona C. Robertson

Abstract The stomatal guard cell regulates gas exchange between the plant and the environment. The movements of the stomata are regulated by a myriad of signals. The signalling pathways regulating stomatal movements have been intensely investigated due to their importance in plant responses to environmental stresses and because transpiration from the stomatal pore is the major route for water flux from the soil to the atmosphere, having consequence for climate models. The ubiquitous second messenger, calcium, is an important regulator of stomatal movements. The role of calcium as a second messenger in abscisic acid-induced stomatal closure is described. The importance of repetitive oscillations in the concentration of cytosolic free Ca^{2+} is discussed. The use of network reconstruction tools and systems approaches to understanding the relationship between calcium signalling and the recently discovered kinase/phosphatase-based ABA signalling cascade is considered.

1 The Stomatal Guard Cell

The stomata are pores in the leaf epidermis that allow gas exchange between the leaf interior and the atmosphere. Movements of the stomatal guard cells alter the size of the pore, primarily to regulate water loss from the plant. Water loss via evapotranspiration is essential for plants because it both cools the leaf and drives the transpiration stream. Before the evolution of guard cells capable of regulating the size of the stomatal pore, plants were only a few millimetres high because water loss above the soil boundary layer was excessive. During times of severe stress, the guard cells close the pore to minimise water loss, and this is at the expense of

A.A.R. Webb (✉) • F.C. Robertson
Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, UK
e-mail: Alex.webb@plantsci.cam.ac.uk

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carbon uptake but offers the opportunity for survival of the plant until the stress is relieved. During more favourable conditions, the size of the aperture of the stomatal pore is continuously altered by the movements of the stomata to optimise the ratio between CO_2 uptake and water loss (Webb 1998, 2003).

Stomatal movements result from changes in the turgor of the guard cell. Increases in turgor cause opening of the stomatal pore due to asymmetrical thickening of the guard cell wall. The changes in turgor are caused by fluxes of water driven by alterations in the osmolyte content of the guard cell vacuole. The principal osmolytes are K, Cl, malate and sucrose (Talbott and Zeiger 1998). Calcium ions are major regulators of ion fluxes in guard cells and for this reason this chapter will focus on the regulation of ion fluxes, whilst recognising also the importance in the changes of metabolism in controlling turgor.

In C3 plants, the circadian clock (Somers et al. 1998; Dodd et al. 2004), blue light signals and possibly auxin promote stomatal opening in the morning. After midday, the circadian clock and darkness promote closure, such that in C3 plants the stomata are open in the light to allow CO_2 uptake and at night they are closed to conserve water (Webb 1998). During times of water deficit stress, abscisic acid (ABA) is produced in the roots and guard cells and accumulation in the guard cells promotes stomatal closure (Okamoto et al. 2009). There is an interaction between the circadian clock and ABA signalling in promoting stomatal closure, with ABA being most effective after midday (Robertson et al. 2009). It is unclear whether this is related to the circadian regulation of ABA signalling intermediates (Love et al. 2004; Dodd et al. 2007).

There has been much focus on the role of changes in the concentration of cytosolic-free Ca^{2+} ($[Ca^{2+}]_{cyt}$) in ABA signalling over the last two decades because an increase in $[Ca^{2+}]_{cyt}$ is one of the earliest events in the guard cell following ABA addition (McAinsh et al. 1990), ABA-induced increases in $[Ca^{2+}]_{cyt}$ appear to be required for the full response of stomata to ABA (Webb et al. 2001; Siegel et al. 2009) and ABA-induced increases in $[Ca^{2+}]_{cyt}$ can be oscillatory (Staxén et al. 1999). A review on this topic is timely because very recently huge advances in identifying the nature of the ABA signalling network have been made, including identification of bona fide receptors (Ma et al. 2009; Park et al. 2009). However, these studies have not clarified the role of $[Ca^{2+}]_{cyt}$ in ABA-induced stomatal closure nor how oscillatory signals are generated.

2 Ion Fluxes and Stomatal Movements

The movements of solutes in and out of the guard cell to drive turgor changes are controlled by the plasma membrane potential. Stomatal opening is achieved by activation of a plasma membrane-bound H^+-ATPase that consumes ATP to pump H^+ out of the cell and energise the membrane (Fig. 1). Stomata can develop plasma membrane potentials as great as −250 mV (cytosolic face of the membrane negative). This hyperpolarisation along with a chemical gradient provides a driving force

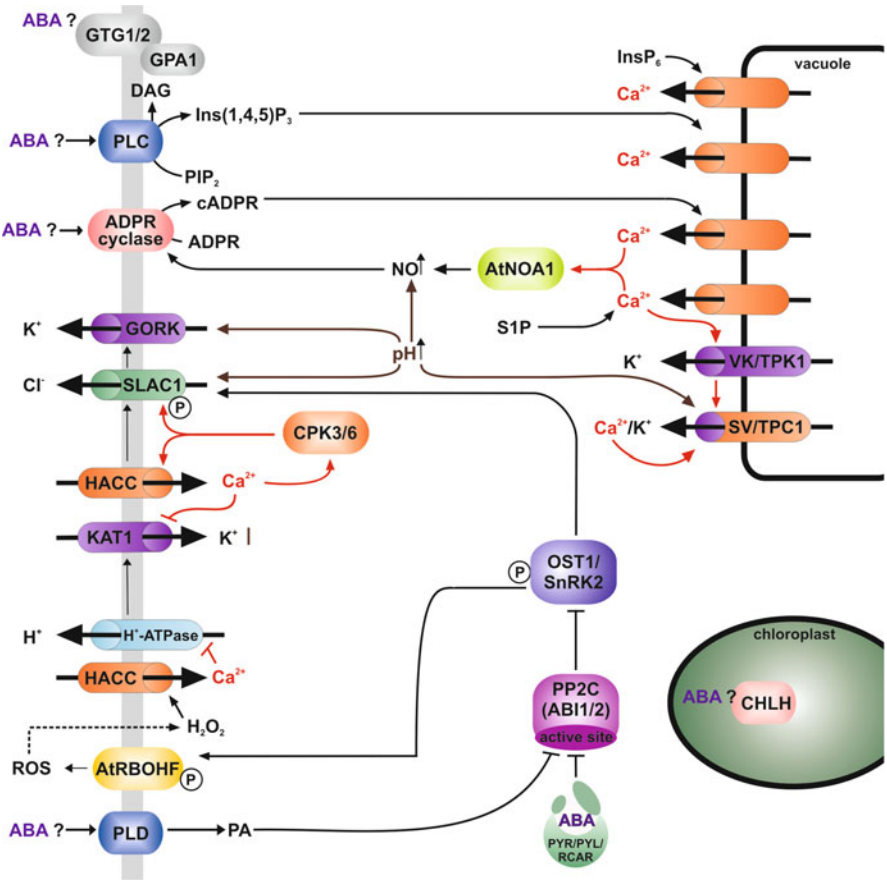

Fig. 1 A schematic representation of the signalling and ionic events associated with stomatal closure. *Arrows* are positive relationships whereas *crossed lines* represent inhibitory events. Ion transporters are represented by *cylinders* (*purple*, K^+ channels and *orange*, Ca^{2+} channels). P represents phosphorylation. Abbreviations are described in the text

for K^+ uptake through voltage-regulated inward K^+ channels that are active at plasma membrane potentials negative of −120 mV (the approximate reversal potential for K^+). In guard cells of Arabidopsis, inward K^+ channel activity is contributed to by at least five Shaker-type K^+ channels, potassium channel in *Arabidopsis thaliana* 1 (KAT1), KAT2, Arabidopsis K transporter 1 (AKT1), AKT2 and *A. thaliana* K^+ rectifying channel 1 (AtKC1) (Very and Sentenac 2003; Fig. 1). Cl^- and malate are the major anions that balance the K^+ charge and contribute to salt accumulation. Malate is synthesised and Cl^- is taken up by poorly characterised transport systems (Barbier-Brygoo et al. 2000). These salts accumulate in the vacuole driving water influx. To bring about stomatal closure, signals such as ABA must both induce efflux of K^+ and anions from the vacuole across the tonoplast, and accompany this with depolarisation of the plasma membrane to values positive of the reversal

potential for K^+, so that K^+ can leave the cell. Ca^{2+} appears to be a major regulator of ion fluxes in guard cells co-ordinating events both at the plasma membrane and the tonoplast (Fig. 1).

3 ABA Receptors and Early Transduction Events

Members of the 14 START protein family known as the [pyrabactin resistance/PYR1-like/regulatory component of ABA receptor 1 (PYR/PYL/RCAR1)] are ABA receptors located in the cytosol (Ma et al. 2009; Park et al. 2009; Fig. 1). ABA is bound in a ligand-binding pocket of PYR/PYL/RCAR resulting in allosteric modification of the receptor that causes a gating loop to "shut", locking ABA into place and allowing binding of protein phosphatase 2C proteins (PP2C) (Melcher et al. 2009; Miyazono et al. 2009; Santiago et al. 2009; Nishimura et al. 2009; Yin et al. 2009; Fig. 1). PP2Cs including abscisic acid insensitive 1 (ABI1) and 2 (ABI2) (Ma et al. 2009; Park et al. 2009) bind the gating loop of the ABA-bound PYR/PYL/RCAR both obscuring the PP2C active site and inhibiting PP2C activity (Melcher et al. 2009; Miyazono et al. 2009; Santiago et al. 2009; Nishimura et al. 2009; Yin et al. 2009). PP2Cs are negative regulators of ABA signalling and inhibition of the PP2C activity is required for ABA signalling to proceed (Gosti et al. 1999). Inhibition of the PP2Cs favours autophosphorylation of members of the SNF1-related protein kinase family (SnRK2). The SnRK2s are a family of plant-specific protein kinases containing 10 members (SnRK2.1–2.10) in Arabidopsis, many of which participate in ABA signalling (Fujii and Zhu 2009). The default state of the SnRK2s is active and they are kept inactive by the dephosphorylating activity of the PP2Cs. In the presence of ABA, the PYR/PYL/RCAR receptors prevent the interaction between the PP2Cs and SnRK2s, favouring autophosphorylation and activation of the SnRK2s (Fujii et al. 2009). The SnRK2s have many downstream targets in ABA signalling and the kinase activity promotes downstream responses. SnRK2 targets include ABA-responsive transcription factors (Fujii et al. 2009) though this might not be required for ABA-induced stomatal closure. SnRK2.6 (also known as *open stomata 1*) is likely to participate in ABA-induced stomatal closure because it phosphorylates *A. thaliana* respiratory burst oxidase protein F (AtRBOHF), an NADPH oxidase that generates reactive oxygen species that result in H_2O_2 formation (Sirichandra et al. 2009; Fig. 1). An increase in H_2O_2 in the cytosol is detected within 30 s of ABA treatment and is thought to increase $[Ca^{2+}]_{cyt}$ by activating hyperpolarisation-activated Ca^{2+} channels (HACC) in the plasma membrane (Pei et al. 2000; Fig. 1). Similarly, a key step in stomatal closure, slow anion channel 1 SLAC1 (SLAC1) activation, is promoted by SnRK2.6/OST1-mediated phosphorylation (Lee et al. 2009; Negi et al. 2008; Vahisalu et al. 2008) and SnRK2.6/OST1 is required for SLAC1 activation (Geiger et al. 2009; Fig. 1). In the absence of ABA, SLAC1 is not phosphorylated because PP2C proteins interact with SnRK2.6/OST1 and block its kinase activity and dephosphorylate the SLAC1 protein (Lee et al. 2009). Additionally, ABA-activated

SnRK2.6/OST1 phosphorylates KAT1 at theonine 306 and this might contribute to KAT1 channel regulation (Sato et al. 2009).

It is possible that other ABA receptor proteins contribute to ABA-induced stomatal closure. A recently proposed G-protein coupled ABA receptor (GCR2) (Liu et al. 2007) is unlikely to be an ABA receptor because the classification of GCR2 as a seven membrane-pass protein typical of the G-protein receptor class appears incorrect, and identification of GCR2 ABA binding activity was overoptimistic and reports of ABA-sensitivity phenotypes in GCR2 knock downs and over expressers irreproducible (Risk et al. 2009). The proposed role of another class of putative G-protein coupled ABA receptor with GTPase activity (GTG1 and GTG2) (Pandey et al. 2009) has been also questioned (Risk et al. 2009). GTG1 and GTG2 were identified on the basis of 65% amino acid sequence similarity to a human orphan G-protein coupled receptor, but the human protein has since been identified as an anion channel (Risk et al. 2009). Additionally, only 1% of the GTG1 protein pool binds ABA, rather than the 1:1 stoichiometry expected of a receptor (Risk et al. 2009).

Mutations in CHLH, which encodes a subunit of magnesium-protoporphyrin-IX chelatase (Mg-chelatase), alter ABA sensitivity (Shen et al. 2006) and ABA binds the C-terminal and not the N-terminal of CHLH in the nM range, with a roughly 1:1 protein:ligand ratio (Wu et al. 2009). This has led to the proposal that CHLH might function as part of a receptor, but the mechanisms by which a chloroplastic-localised enzyme involved in chlorophyll biosynthesis might function as an ABA receptor are unclear (Fig. 1).

4 The ABA Ca^{2+} Signalling Network in Guard Cells

The recently established signalling cascade by which ABA-bound PYR/PYL/RCAR binds and inhibits PP2Cs allowing SnRK2s to autophosphorylate and phosphorylate target proteins is central to stomatal responses to ABA. The OST1-induced activation of AtRBOHF to generate H_2O_2 (Sirichandra et al. 2009) and increase $[Ca^{2+}]_{cyt}$ by activating HACCs in the plasma membrane (Pei et al. 2000) provides a link to another key process in stomatal movements, because alterations in $[Ca^{2+}]_{cyt}$ are also pivotal for the regulation of the channel activities underlying stomatal movements (Fig. 1). ABA-induced stomatal closure is severely compromised in guard cells in which changes in $[Ca^{2+}]_{cyt}$ are prevented by artificial buffering (Webb et al. 2001; Siegel et al. 2009). Elevated $[Ca^{2+}]_{cyt}$ inhibits the plasma membrane H^+-ATPase, preventing further hyperpolarisation of the membrane (Kinoshita et al. 1995); inhibits the inward K^+ channel activity (Schroeder and Hagiwara 1989) and promotes anion efflux across the plasma membrane and depolarisation by activating SLAC1 (Negi et al. 2008; Vahisalu et al. 2008; Fig. 1). SLAC1 is relatively voltage insensitive, permitting SLAC1 to remain active as the plasma membrane depolarises, making SLAC1 suited to carry sustained efflux of anions out of the cell. This sustained efflux of anions is the critical event in stomatal

closure because it brings the plasma membrane potential positive of the reversal potential for K^+, permitting K^+ efflux from the cell. At values positive of approximately −80 mV, guard cell outward rectifying K channel (GORK), a shaker-type channel, is activated to allow K^+ efflux from the cell down the chemical gradient (Hosy et al. 2003; Fig. 1). Thus, the efflux from the guard cell of anions is directly Ca^{2+} dependent and the efflux of cations is indirectly Ca^{2+} dependent. In addition to regulating ion transport at the plasma membrane, ABA-induced elevations in $[Ca^{2+}]_{cyt}$ are also responsible for promoting K^+ efflux from the vacuole to the cytosol by activating TPK1/VK (Gobert et al. 2007) and the slow vacuolar/two pore channel 1 (SV/TPC1) (Peiter et al. 2005; Fig. 1).

The events downstream from Ca^{2+} leading to channel regulation are not well known. It is likely that regulation of channel activity by $[Ca^{2+}]_{cyt}$ is not mediated by SnRK2.6/OST1 activity because there are multiple phosphorylation sites in KAT1, in addition to those regulated by SnRK2.6/OST1 (Sato et al. 2009) and the calcium-dependent protein kinase 3 and 6 (CPK3/6) are required for the $[Ca^{2+}]_{cyt}$ regulation of SLAC1 (Mori et al. 2006).

5 Mechanism by which ABA Increases $[Ca^{2+}]_{cyt}$ in Guard Cells

Multiple pathways lead to an elevation in $[Ca^{2+}]_{cyt}$ in guard cells. ABA-induced increases in $[Ca^{2+}]_{cyt}$ can be transitory, sustained or oscillatory. The nature of the ABA-induced $[Ca^{2+}]_{cyt}$ signal depends partially on the plasma membrane potential, with oscillations occurring in more guard cells when the membrane is hyperpolarised (Staxén et al. 1999). $[Ca^{2+}]_{cyt}$ is maintained at around 100 nM against a step concentration gradient, with $[Ca^{2+}]$ in the extracellular medium and vacuolar lumen in the mM range and in the ER about 1.5 μM. These large gradients provide the opportunity for multiple Ca^{2+} influx routes that have different regulatory properties. By combinatorial activation of the suite of Ca^{2+} influx pathways the guard cell generates complex temporal and spatial patterns of $[Ca^{2+}]_{cyt}$ dynamics.

At least two types of channel are involved in mediating Ca^{2+} influx across the plasma membrane. The earliest electrical event following ABA application is the activation of a depolarising "leak" conductance that might represent in part non-specific cation channels capable of supporting Ca^{2+} influx (MacRobbie 1992). ABA also activates HACCs (Hamilton et al. 2000). The electrical properties of the HACC make it well suited to supporting sustained Ca^{2+} influx into the cytosol. The HACC is active at hyperpolarised plasma membrane potentials because ABA must be able to initiate the closing of open stomata and thus requires the HACC to be activated at the extreme hyperpolarised potentials of a guard cell of an open stoma. In the absence of ABA, the activation potential of the HACC is approximately −150 mV but when ABA is present, the activation potential shifts positive to around −50 mV. This shift positive in the HACC activation potential by ABA permits the HACC

to remain active during SLAC1-induced depolarisation of the plasma membrane. In addition to shifting the activation potential positive, ABA also increases current through the HACC (Hamilton et al. 2000). A feed forward loop appears to potentiate HACC activation because $[Ca^{2+}]_{cyt}$ sensing by CPK3 and CPK6 is required for HACC activation (Mori et al. 2006; Fig. 1). A negative feedback must also be present because the open probability of the channel oscillates over time (Hamilton et al. 2000), which might be responsible for stimulus-induced oscillations in Ca^{2+} influx across the plasma membrane (McAinsh et al. 1995).

There are at least four routes for Ca^{2+} entry across the tonoplast. SV/TPC1 is ubiquitous in plant cells and is a plant-specific system for Ca^{2+}-induced Ca^{2+} influx into the cytosol (Fig. 1). TPC1 has two EF hands that act as Ca^{2+} sensors (Peiter et al. 2005). SV/TPC1 is activated by increases in $[Ca^{2+}]_{cyt}$ over 300 nM; however, activation also requires depolarisation of the tonoplast, which is brought about by Ca^{2+}-induced activation of TPK1/VK (Gobert et al. 2007; Fig. 1). The Ca^{2+}-activated K^+ efflux through TPK1/VK, along with sensitisation by calmodulin is proposed to sufficiently depolarise the tonoplast to allow SV/TPC1 to be active (Ward and Schroeder 1994).

$Ins(1,4,5)P_3$ is thought to activate Ca^{2+} influx from both the vacuole and ER through ligand-gated Ca^{2+} channels, though the molecular identity of these channels in plants is unknown (Fig. 1). ABA activates phospholipase C (PLC) in guard cells (Lee et al. 1996; Fig. 1) to cleave phosphatidylinositol (4,5)bis phosphate (PIP_2) to release the inositol(1,4,5)trisphosphate [$Ins(1,4,5)P_3$] head group that increases $[Ca^{2+}]_{cyt}$ (Gilroy et al. 1990). However, the mechanism by which PLC is activated by ABA is not known. The properties of $Ins(1,4,5)P_3$-mediated increase in plants have been extensively studied in red beet vacuoles and show similarities to $Ins(1,4,5)P_3$-mediated Ca^{2+}-release mechanisms in mammals, the Kd for $Ins(1,4,5)P_3$ is 0.2–1 μM and $Ins(1,4,5)P_3$-mediated Ca^{2+}-release is sensitive to heparin and specific for $InsP_3$ over other inositol phosphates, e.g. $InsP_4$ (Webb et al. 1996). However, unlike mammalian systems, high $[Ca^{2+}]_{cyt}$ does not inhibit the $Ins(1,4,5)P_3$-mediated Ca^{2+}-release in plants (Webb et al. 1996). Inositol hexakisphosphate ($InsP_6$) also releases Ca^{2+} from the vacuole to elevate guard cell $[Ca^{2+}]_{cyt}$ through a pathway separate to that by which $Ins(1,4,5)P_3$ releases Ca^{2+}(Lemtiri-Chlieh et al. 2003; Fig. 1).

Cyclic adenosine diphosphate ribose (cADPR) causes Ca^{2+} influx from both the vacuole (Leckie et al. 1998) and ER (Navazio et al. 2001) in plants (Fig. 1). ABA increases the production of cADPR (Sánchez et al. 2004) and ABA-induced stomatal closure is inhibited by antagonists of cADPR production and signalling (Leckie et al. 1998). Injection of cADPR into the cytosol of guard cells elicits low amplitude oscillations in $[Ca^{2+}]_{cyt}$, consistent with inhibition of cADPR-mediated Ca^{2+} release by elevated $[Ca^{2+}]_{cyt}$ (Leckie et al. 1998). cADPR is produced by ADPR cyclase activity which is present in plants (Dodd et al. 2007; Fig. 1) but neither the ADPR cyclase nor the molecular targets for cADPR have been identified in plants. In animals, it has been proposed that the ryanodine receptor might be a cADPR-gated Ca^{2+} channel (Galione et al. 1991). Recent work also suggests that cADPR might alter $[Ca^{2+}]_{cyt}$ by affecting the activity of Ca^{2+}-ATPases responsible

for removing Ca^{2+} from the cytosol (Yamasaki-Mann et al. 2009). In plants, cADPR might act in a feed forward loop, in conjunction with nitric oxide (NO), to increase $[Ca^{2+}]_{cyt}$ (Fig. 1). Ca^{2+} can activate nitric oxide associated 1 in *A. thaliana* to increase NO in the cell (Guo et al. 2003; Gonugunta et al. 2008) and NO, in turn, increases $[Ca^{2+}]_{cyt}$ through a cADPR-dependent pathway (Garcia-Mata et al. 2003). Sphingosine 1-phosphate (S1P), a potential signalling intermediate, is also capable of inducing oscillatory $[Ca^{2+}]_{cyt}$ signals in guard cells, though the role of S1P and its targets is not well understood (Ng et al. 2001; Fig. 1).

6 The Role of pH

In the first 2 min following ABA application, a rise in cytosolic pH from approximately 7.4 to 7.7 can be detected (Armstrong et al. 1995). The rise in pH is intriguing and an underexplored phenomenon. Neither the mechanisms by which pH is increased nor the immediate downstream targets for pH signalling are known. However, an increase in pH is essential for the activation of the outward K^+ flux and inhibits inward K^+ channels (Grabov and Blatt 1997; Fig. 1).

7 Reconstruction of Ca Signalling Networks in Guard Cells

We propose in Fig. 1 a network structure for ABA signalling in stomatal guard cells based on our interpretation of the literature. In recent years, attempts have been made to obtain more formal, unbiased constructions of the ABA signalling network. A common feature of network reconstruction approaches is that an assembly of putative components must first be obtained using literature surveys, genetics and/or "omics". There is an extensive literature on the physiology of the guard cell that is a resource for network reconstruction (Hetherington and Woodward 2003; Li et al. 2006). Similarly, extensive forward and reverse genetic studies have identified many components in ABA signalling. Transcriptomic and proteomic analyses of guard cells have been hampered because the guard cells make up a small proportion of the leaf and obtaining sufficient quantity of isolated pure guard cells without major changes in transcription or translation is technically demanding (Gardner et al. 2009). Pure preparations of guard cells can be obtained by protoplasting because the extremely thick guard cell wall allows separation of guard from other cells using differential digestion with cellulolytic enzymes (Gardner et al. 2009). Protoplasting of guard cells can take 5–8 h, resulting in major transcriptional/translation changes due to cell wall digestion and osmotic stress. The induction of stress-induced genes can be prevented by including transcriptional inhibitors in the preparation media but this prevents assaying the abundance of transcripts that are rapidly turned over (Leonhardt et al. 2004). ABA-regulated genes in guard cell protoplasts (GCP) fall into two categories,

genes involved in cell protection including enzymes required for the biosynthesis of osmoprotectants (sugars) and proteins that may protect macromolecules and membranes (e.g. *late embryogenesis abundant* and chaperones), and those involved in signal transduction (Leonhardt et al. 2004). A similar protoplasting approach has been used to obtain a proteome for the guard cell (Zhao et al. 2008), although few of the proteins implicated previously in ABA signalling were detected in the guard cell proteome. Whether the failure to detect the majority of ABA-signalling elements in the GCP proteome reflects technical barriers or the possibility that these components are induced under specific conditions remains to be determined (Zhao et al. 2008).

The transcriptomic and proteomic data for guard cells have yet to be formulised into coherent networks. The only formal dynamical model of ABA-induced stomatal closure at the time of writing is a Boolean network based on interactions derived from a literature search (Li et al. 2006). The network was formed on the basis of experimental, genetic and pharmacological inference of relationships between components. The network was reconstructed by assigning only two forms to the edges, activating or inhibitory and the nodes represent components of the ABA signalling pathway. The network structure was based on intuitive inference rules to form the sparsest graphical representation of the network. Path analysis of the model suggested that the pH-dependent and Ca^{2+}-dependent signalling pathways are independent, consistent with earlier experimentation (Grabov and Blatt 1997; Li et al. 2006).

To allow dynamic analyses of the Li et al. (2006) model, the input (ABA), output (stomatal closure) and nodes (signalling components) were permitted unitary states of either 0 (e.g. no ABA input = 0, stomata open output = 0) or 1 (ABA present input = 1 and stomata closed output = 1) and nodes were permitted to sequentially regulate each other. The state changes of the nodes was governed by the state of its regulators (upstream nodes) and defined using combinations of Boolean (AND, OR, NOT) logic gates based on experimental evidence. To perform dynamical modelling, simulations were run with each node assigned a random state, with the exception of the input (ABA), which was defined as 1, and the output (stomatal closure) which was initially assigned 0. The simulations were run in time steps allowing sequential changes in node state. During one time step, all nodes were visited at random once only and the state changed according to the upstream nodes. When ABA = 1, after eight time steps all simulations resulted in closed stomata. However, guard cells have a distribution of apertures, therefore the model assesses the probability that each stomata in the population exhibits a significant change in aperture. The dynamical modelling predicted that in the presence of ABA, some components reach steady state (e.g. OST1 and PLC) whereas some components oscillate including, $[Ca^{2+}]_{cyt}$, Ca^{2+}-ATPase activity, NO, K^+ efflux from the vacuole to the cytosol, and rapidly activating K^+ efflux across the plasma membrane (Li et al. 2006).

The effects of mutations and pharmacological perturbations were simulated by removal of nodes and subsequent dynamic simulations. Consistent with earlier pharmacological and later genetic studies, loss of plasma membrane depolarisation due to the disruption of anion efflux caused ABA insensitivity (Li et al. 2006; Vahisalu et al. 2008). Whereas, loss of phospholipase D (PLD) or its product

phosphatidic acid (PA) (Zhang et al. 2004), loss of sphingosine kinase activity or its product S1P, loss of G protein alpha subunit activity (GPA1; Pandey et al. 2009), loss of K^+ efflux through slowly activating K^+ channels at the plasma membrane or loss of ABA-induced pH increase reduced ABA sensitivity (Li et al. 2006).

Some simulated perturbations affected the number of time steps required for stomata to be closed (Li et al. 2006). Loss of an increase in $[Ca^{2+}]_{cyt}$ or of the production of H_2O_2 led to ABA hyposensitivity (slower than wild-type response) consistent with the partial inhibition of ABA-induced stomatal closure in Arabidopsis when $[Ca^{2+}]_{cyt}$ was buffered to resting levels (Siegel et al. 2009). In contrast, the disruption of ABI1 or of the Ca^{2+}-ATPase(s) lead to ABA hypersensitivity (faster than wild-type response). The prediction concerning ABI1 is consistent with the proposed PYR/PYL/RCAR-PP2C-SnRK2 cascade.

The effect of buffering an increase in $[Ca^{2+}]_{cyt}$ was predicted to be conditional, because an increase in $[Ca^{2+}]_{cyt}$ became required for stomatal closure when pH changes, K^+ efflux across the membrane or the S1P and PA pathways were perturbed (Li et al. 2006). This again is consistent with the experimental data, because in high external KCl that perturbs K^+ and Cl^- efflux, stomata of *Commelina communis* were essentially ABA-insensitive when $[Ca^{2+}]_{cyt}$ was buffered at resting values (Webb et al. 2001). The model of Li et al. (2006) has been validated by the experimental data and provides a good basis for a formal description of ABA signalling in guard cells. The use of Boolean rules avoids the problems of assigning an enormous set of parameters to the ordinary differential equations commonly used to describe biological processes. However, the model has some omissions that necessitate further model development, in particular the role of the vacuole ought to be considered.

8 Temporal Oscillations of $[Ca^{2+}]_{cyt}$ in Guard Cells

ABA, high external $[Ca^{2+}]$ and low external $[K^+]$ induce sustained oscillations in $[Ca^{2+}]_{cyt}$ (McAinsh et al. 1995; Staxén et al. 1999; Allen et al. 1999). The oscillation pattern and period depends on the strength and type of stimuli applied (McAinsh et al. 1995; Staxén et al. 1999; Allen et al. 1999) leading to the suggestion that information about stimulus type and strength might be encoded in the $[Ca^{2+}]_{cyt}$ oscillation (McAinsh et al. 1995). A non-oscillatory rise in $[Ca^{2+}]_{cyt}$ is sufficient to initiate closure (Gilroy et al. 1990) and the rate of initial stomatal closure following stimulus application is not affected by the period and amplitude of the circadian oscillations of $[Ca^{2+}]_{cyt}$ (McAinsh et al. 1995; Mori et al. 2006). Following the initial rapid closure, which typically lasts 0.5–1 h, the guard cell goes through a second phase of adjustment in which either the stomata remain closed or reopen to a new set point. It is in this second phase of sustained readjustment of stomata that regulation is coupled to the period and duration of the $[Ca^{2+}]_{cyt}$ oscillations (Allen et al. 2001). Oscillatory $[Ca^{2+}]_{cyt}$ signals are important for the normal functioning of stomata because the *det3* mutation that results in reduced

expression of the V-ATPase and reduced tonoplast energisation interferes with $[Ca^{2+}]_{cyt}$ oscillations and sustained stomatal closure (Allen et al. 2000). The mechanisms that generate $[Ca^{2+}]_{cyt}$ oscillations are stimulus-specific because *det3* abolishes oscillations of $[Ca^{2+}]_{cyt}$ and sustained closure in response to elevations in external $[Ca^{2+}]$ and H_2O_2 but is without effect on $[Ca^{2+}]_{cyt}$ oscillations and closure induced by cold and ABA (Allen et al. 2000). Short-term closure caused by an increase in $[Ca^{2+}]_{cyt}$ and long-term inhibition of reopening by $[Ca^{2+}]_{cyt}$ oscillations are genetically separable. In the *cpk3cpk6* double mutant, the initial closure response to artificial $[Ca^{2+}]_{cyt}$ oscillations is inhibited but the mutations were without effect on the long-term closure caused by artificial $[Ca^{2+}]_{cyt}$ oscillations (Mori et al. 2006). The physiological and genetic data suggest that there are at least two Ca^{2+}-activated signalling networks in guard cells, one which is short term and causes an immediate regulation of ion fluxes to bring about closure. The second pathway is responsive to the temporal dynamics of the $[Ca^{2+}]_{cyt}$ signal and has more long-term consequences for stomatal movements. Little is understood about this signalling cascade that responds to oscillatory $[Ca^{2+}]_{cyt}$ signals, nor how the signals are generated in a stimulus-specific manner.

9 Conclusions

In 2009, there was an explosion of information concerning the signalling network by which ABA closes stomata. We have learnt that the PYR/PYL/RCAR family of ABA receptors regulates a PP2C-SnRK2 cascade targeting SLAC1 and AtRBOHF activation. This provides an apparently nearly complete and elegantly simple picture of the major mechanisms of ABA-induced stomatal closure. This draws together many experimental findings over the previous two decades into a coherent network. However, as always with science, one answer always leads to another question, "what is the role of $[Ca^{2+}]_{cyt}$?" because an increase in $[Ca^{2+}]_{cyt}$ alone is sufficient to close stomata, directly regulates the inward K^+ current and activates anion efflux. The effects of $[Ca^{2+}]_{cyt}$ are in part mediated by CPK3 and 6, but these are not responsible for decoding $[Ca^{2+}]_{cyt}$ oscillations. The challenges now are to fathom how $[Ca^{2+}]_{cyt}$ integrates with the PYR/PYL/RCAR-PP2C-Snrk2 cascade, identify how $[Ca^{2+}]_{cyt}$ regulates channels and identify the mechanisms for decoding $[Ca^{2+}]_{cyt}$ oscillations.

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Stimulus Perception and Membrane Excitation in Unicellular Alga *Chlamydomonas*

Kenjiro Yoshimura

Abstract *Chlamydomonas* cells display various Ca^{2+}-dependent behavioral responses against environmental stimuli to find a better place for proliferation. The cells show phototaxis to move toward a light condition suitable for photosynthesis and also display photophobic response to avoid excessive light. The flagellar motility during phototaxis and photophobic response is controlled by changes in intraflagellar Ca^{2+} concentration. Ca^{2+} influx on photophobic response is brought about by voltage-dependent calcium channel, CAV2. Avoiding reaction, which occurs on collision to obstacles, is triggered by mechanosensitive channel TRP11, a member of transient receptor potential channels, subfamily V. Elimination of the CAV2 localization in the flagellar proximal region prevents untimely activation of Ca^{2+}-dependent flagellar excision machinery at flagellar base. By contrast, false activation TRP11 by the flagellar bending motion is kept to minimum by targeting TRP11 to the proximal region of flagella. A set of Ca^{2+}-dependent processes performed by flagella is coordinated by various ion channels that show specific distribution along the length of flagella.

1 Introduction

Although land plants have no chocice other than surviving at the very place where seeds germinate, unicellular algae have a chocie to migrate to a place suitable for survival. Unicellular algae display various types of responses against vital factors such as light, oxygen, carbon dioxide, and nutrients. Cells also respond to gravity and show noticeable vertical distribution probably because the concentrations of gas and nutrition vary with the depth in water. The tendency to move toward the source of these factors is called positive taxis. Negative taxis represents the

K. Yoshimura (✉)
Department of Biology, University of Maryland, College Park MD, 20742, USA
e-mail: kenjiro@mbb.nifty.ne.jp

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tendency of migrating away from harmful factors. To achieve this directional movement, cells are equipped with mechanisms for detecting the direction of stimuli and steering the swimming direction accordingly.

Chlamydomonas, a unicellular green alga, displays marked phototaxis and photophobic response, and thus has been used as a model organism for the study of photoresponses. The availability of the genomic database and various molecular biological techniques has supported the research (Stern et al. 2008). These tools have also provided a breakthrough in the study on the mechanoresponses. In this article, the mechanisms of photoresponses and mechanoresponses in *Chlamydomonas* are reviewed with special emphasis on the calcium-signaling mechanisms in the stimulus perception and motility regulation.

2 Photoresponse of *Chlamydomonas*

In a natural fresh water environment, in which dissolved carbon dioxide is the only carbon source, it is vital for *Chlamydomonas* cells to migrate to a light environment adequate for photosynthesis. Stronger is not always better; although insufficient light intensity of light would result in poor growth, excess light intensity would induce photoinhibition of photosynthesis. Consequently, *Chlamydomonas* cells swim not only toward light but also away from excessive light depending on the light intensity and cell condition. The directional movement toward or away from light is referred to as positive and negative phototaxis (Fig. 1a). The sign (direction)

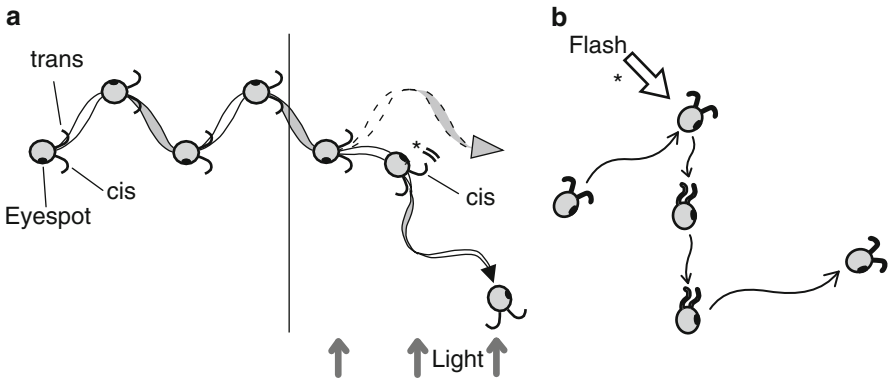

Fig. 1 *Response of Chlamydomonas cells* to *photostimulation*. (**a**) Phototaxis. The swimming direction gradually changes toward the light incident from the *bottom* of this figure. A large turn (*asterisk*) toward the light occurs when the eyespot is directed away from the light source. (**b**) Photophobic response. A flash light (*asterisk*) induces a temporal backward swimming, in which the flagellar bending pattern changes from the breaststroke-like pattern to the undulatory pattern

of phototaxis is under complex influences of photosynthetic, circadian, and growth conditions, but how these factors determine the direction is not fully understood.

Whereas phototaxis is a response continuous light, a sudden increase in light intensity evokes photophobic response, in which cells swim backward for about 0.5 s (Fig. 1b). These responses are triggered by the photoreceptor current at the eyespot and, when the membrane depolarization exceeds a threshold level, an action potential is generated at flagella by voltage-dependent calcium channels (Harz and Hegemann 1991) (Fig. 2b).

Two flagella at the anterior end usually beat in a breaststroke-like pattern. Because of slight difference in the bending pattern between the two flagella, cells swim in a helical path while rotating the cell body in a manner such that the eyespot,

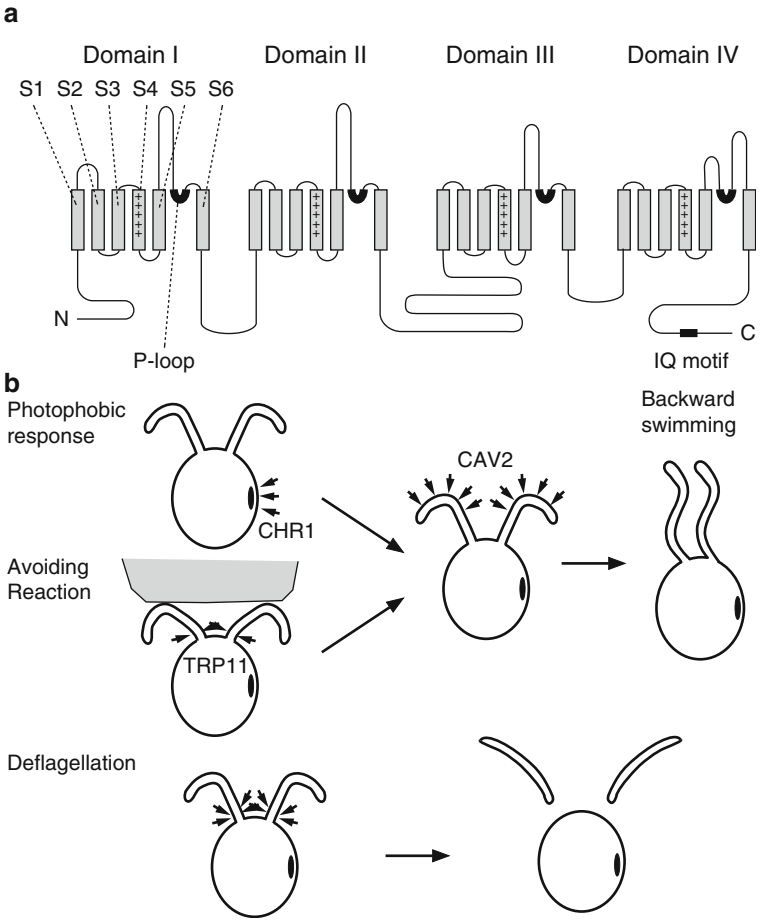

Fig. 2 Structure and localization of flagellar ion channels. (**a**) Diagram of the molecular structures of the CAV2 voltage-dependent calcium channel. (**b**) Localization of Ca^{2+} influx during photophobic response, avoiding reaction, and deflagellation. *Arrows* indicate the Ca^{2+} influx

which is located on the equator of the cell body, is always directed away from the helix axis (Isogai et al. 2000) (Fig. 1a). Although this outward orientation of eyespot is dominant (but only about 80% in our experiment) in cells showing positive phototaxis, inward orientation is more often observed in cells showing negative phototaxis (but only about 60%), indicating that the orientation of eyespot relative to the helix axis is related weakly the sign of taxis. Because the eyespot is the photoreceptor for phototaxis and is most sensitive to the light incident from the direction perpendicular to the eyespot membrane, the light intensity perceived by the eyespot increases when the eyespot a points toward light source and decreases when it points toward the opposite direction. Therefore, cells seem to use the eyespot as a rotating radar antenna and detect the position of the light (Foster and Smyth 1980). The photoreceptor is tuned to the light intensity change at the frequency of the cell body rotation to exclude the fluctuation of light intensity from other sources (Yoshimura and Kamiya 2001).

The plasma membrane at the eyespot consists of photoreceptor proteins, channelopsins (Nagel et al. 2002, 2003; Sineshchekov et al. 2002; Suzuki et al. 2003). Channelopsins are peculiar photoreceptor proteins in that they are photoreceptors as well as ion channels. Two types of channelopsins, CHR1 and CHR2, are expressed at the eyespot; CHR1 passes cations such as Ca^{2+}, Na^{+}, K^{+}, and H^{+} and is responsible for the photorespouses. The chromophore of the channelopsins is all-*trans* retinal that is oriented parallel to the membrane and thus is similar to archeal-type photoreceptors (Foster et al. 1984; Yoshimura 1994).

The eyespot is observed as a red dot under light microscope, and this color originates from carotenoid granules. The granules are layered at a regular spacing and thereby function as a quarter wave stack reflector (Foster and Smyth 1980, Kreimer and Melkonian 1990). Because this "reflector" is placed just behind the photoreceptor membrane, the light incident from the front of the eyespot passes the plasma membrane twice thus and the light from the back of eyespot is eliminated. The eyespot is most sensitive to the light incident from the front of the eyespot (Harz et al. 1992).

On phototaxis, cells steer the swimming direction by beating one flagellum stronger than the other indicating that the motility of the two flagella of *Chlamydomonas* are regulated independently. To discriminate the two flagella, the flagellum close to and the other one far from the eyespot are referred to as *cis*- and *trans*-flagellum, respectively. Cells that direct the eyespot away from the helix axis should beat the *cis*-flagellum stronger than the *trans*-flagellum. Phototactic turn begins the eyespot points toward the light source, the helical path of the swimming track becomes straight in this initial stage without a significant turn toward the light source (Fig. 1a) (Isogai et al. 2000). This straight path indicates that the force by the two flagella has become comparable. A large turn toward the light source occurrs when the eyespot is directed away from the light source indicating that the *cis*-flagellum beats much stronger than does the *trans*-flagellum. This observation demonstrates that the balance of the force generated by the two flagella changes with the light incident on the eyespot.

The dominance of *cis*- and *trans* flagella is controlled by submicromolar concentrations of intraflagellar Ca^{2+} (Kamiya and Witman 1984). When the cells are demembranated by detergent, the flagella become immotile, but resume motility upon addition of ATP. When the Ca^{2+} concentration of the reactivation solution is 10^{-8} M or less, the movement of *cis*-flagella is dominant over *trans*-flagella, and thus the cells swim in a circular path with the *cis*-flagella away from the center of rotation. In contrast, at Ca^{2+} concentration of 10^{-6} M, *trans*-flagella are dominant over *cis*-flagella. On turn toward light during phototaxis (asterisc in Fig. 1a) the *cis*-flagellum is more active than the *trans*-flagellum and the Ca^{2+} concentration is 10^{-8} M or less. This unusually low Ca^{2+} concentration is possible to be brought about by hyperactivation of Ca^{2+} pump and/or by the closure of background Ca^{2+} leakage into cells.

Many questions, however, still remain to be answered regarding the Ca^{2+}-dependent control of flagellar dominance. For example, the source of Ca^{2+} has not been established; several studies have shown that phototaxis is inhibited by the depletion of Ca^{2+} from solution and by the addition of calcium channel blockers, but the effect is possible to be due to the inhibition of photoreceptor current rather than Ca^{2+} influx at flagella. Moreover, *ptx1* and *lsp1*, which do not switch the dominance of flagellar motility at submicromolar Ca^{2+} concentrations as examined with demembranated cells, still display directional swimming against light although the magnitude is reduced (Okita et al. 2005). Interestingly, these mutants do not display phototaxis as a population because a half of the population shows positive phototaxis and the other half shows negative phototaxis. Therefore, Ca^{2+} at submicromolar concentration regulates the major part of flagellar dominance and determines the sign of phototaxis but there is also a Ca^{2+}-independent mechanism for the regulation of flagellar dominance.

During backward swimming in photophobic response, cells change the flagellar bending mode from a breast stroke pattern (forward mode) to an undulatory pattern (reverse mode), in which flagella propagate S-shaped waves toward the tip of flagella. The experiments using demembranated cells have demonstrated that the conversion between the forward and reverse modes occurs at Ca^{2+} concentrations from 10^{-6} to 10^{-4} M (Hyams and Borisy 1978, Bessen et al. 1980, Goodenough 1983). Thus, the intraflagellar Ca^{2+} concentration should be increased above 10^{-6} M during photophobic response. The Ca^{2+} influx through the voltage-dependent calcium channels elevates the intraflagellar Ca^{2+} concentration and switches the flagellar bending pattern (Yoshimura et al. 1997). The molecular entity and the localization of the voltage-dependent calcium channels will be discussed below.

3 Mechanoresponse of *Chlamydomonas*

The *Chlamydomonas* photoreceptor can detect the light intensity perpendicular to the swimming direction, but cannot "see" the obstacles ahead in the swimming direction. Thus, cells swim forward until they collide against obstacles. On

collision, cells temporarily swim backward to move away from the obstacle and then resume forward swimming in a new direction. This "avoiding reaction" of *Chlamydomonas* can be observed under microscope when the focus is adjusted close to the surface of slide glass, to which cell collide. Historically, the membrane excitation during avoiding reaction has been demonstrated beautifully with on *Paramecium*, to wich electrophysiology is applicable, but the entities of the ion channels responsible for the response have not been unveiled.

Because the time course and the change in the flagellar bending pattern during avoiding reaction are similar to those in photophobic response, the mechanisms for the generation of action potential and the Ca^{2+}-dependent conversion of flagellar bending mode are probably shared between these responses. Mechanoreceptor potential triggers the action potential in the case of avoiding reaction instead of photoreceptor potential (Fig. 2b). The mechanosensitive channels that generate the mechanoreceptor potential will be described below.

Another response to mechanical stimuli is the augmentation of cell motility. When an aliquot of cell suspension is dropped on a slide glass by pipette and a cover slip is put on the droplet, cells swim vigorously for the first few minutes, after which the swimming speed decreases to a steady-state level. The initial increase in the flagellar motility is due to the mechanical agitation of pipetting the cell suspension and putting a cover slip onto the cell suspension (Wakabayashi et al. 2009). This response is generated by the influx of extracellular Ca^{2+} through mechanosensitive channels, but the depolarization is probably not large enough to trigger the action potential. A small elevation in intraflagellar Ca^{2+} concentration possibly results in an increase in flagellar beat frequency without changing the bending pattern to the reverse mode. More extensive mechanical stimulation by pipetting evokes repetitive reversals of swimming direction that take place at about 0.5 Hz (Yoshimura 1996). *Spermatozopsis similis* also display Ca^{2+}-dependent elevation in swimming speed and backward swimming on mechanical stimulation (Kreimer and Witman 1994).

Chlamydomonas cells and accumulate toward the water surface when cell suspension is left standing. Although the accumulation toward the water surface can occur due to the combination of taxis against light, oxygen, and gravity, cells still accumulate close to the water surface, even if they are enclosed in airtight chamber and illuminated with red light, by which phototaxis does not occur. Thus, cells indeed show negative gravitaxis, a tendency to swim in the direction opposite to the gravity (Bean 1977). By contrast, immobile cells sediment to the bottom because the specific gravity of *Chlamydomonas* cells is larger than the medium (approximately 1.04 g cm^{-3}).

It has not been established whether gravitaxis occurs as a physiological response to gravity upward swimming is possible to occur through physical properties of cells without any physiological responses to gravity. In fact, immobilized cells sink with the anterior ends upward suggesting that the swimming direction of motile cells would reorient upward. The anterior ends of immobilized cells turn upward possibly because the center of gravity is located posterior to the center of buoyancy and because the anterior part, where flagella are present, has larger viscous drag

against sedimentaion than the posterior part. However, inconsistent with the former explanation, cells placed in the medium with high specific gravity display positive gravitaxis and swim downward (Yoshimura unpublished data). This observation rather suggests that *Chlamydomonas* cells detect the cell's passive movement relative to medium (i.e., sinking and floating) but not the gravity itself. Yet another result that indicates physical characteristics are not the only factor for gravitaxis is that *gtx*1 and *gtx*2 mutants, whose physical characteristics are normal show impaired gravitaxis, whose (Yoshimura et al. 2003). Membrane excitability probably affects gravitaxis because *ptx*3, *gtx*1, and *gtx*2, which have defect in membrane excitability, show reduced gravitaxis. The influence of background light on gravitaxis also supports the involvement of physiological process (Sineshchekov et al. 2000). Therefore, it is likely that the physical mechanism, if present, does not play a major role in *Chlamydomonas* gravitaxis, but some physiological mechanisms are responsible for gravitaxis.

How the cells steer the swimming direction during gravitaxis is yet to be explored. The control of the flagellar dominance at submicromolar Ca^{2+} concentration, which mainly controls turning of swimming direction in phototaxis, is probably not required in gravitatis because gravitaxis occurs in the absence of extracellular Ca^{2+} and in the *ptx*1 mutant, which has defect in the Ca^{2+}-dependent control of the balance of the flagellar force generation (Kam et al. 1999).

4 Calcium Channels of *Chlamydomonas*

The Ca^{2+} influx into flagella during the flagellar waveform conversion is produced by voltage-dependent calcium channels. Several mutants deficient in generating the flagellar current have been isolated. Among them, *ptx2* and *ptx8* do not produce flagellar current, and display neither phototaxis nor photophobic response (Pazour et al. 1995). In contrast, *ppr1*, *ppr2*, *ppr3*, and *ppr4* show normal phototaxis but no photophobic response (Matsuda et al. 1998). The presence of two distinct phenotypes of flagellar-current-deficient mutants suggests that there are two types of calcium channels: the first type is activated by small depolarization and generates graded depolarization; the second type is activated by larger depolarization and generates all-or-none action potential with the help of the first type. It is possible that the first type is used in both phototaxis and photophobic response, and the second type is used exclusively in photophobic response; *ptx2* and *ptx8* are probably deficient in the first type, and *ppr1*, *ppr2*, *ppr3*, and *ppr4* are deficient in the second type.

The gene responsible for *ppr2* mutation is the α_1 subunit of a voltage-dependent calcium channel, CAV2 (Fujiu et al. 2009). CAV2 has domain structure conserved in voltage-dependent calcium channels, which are comprised of four homologous repeat domains (domain I, II, III, and IV) each with six transmembrane segments: S1, S2, S3, S4, S5, and S6 (Fig. 2a).

The pore of voltage-dependent calcium channels is generally made up with transmembrane segments, S5 and S6 (Hille 2001). P-loops, which connect S5 and S6 at the extracellular side, comprise the selectivity filter and have glutamine residues (EEEE motif) that are essential to Ca^{2+} selectivity. Sodium channels have different amino acids at the corresponding position (DEKA motif). The cytoplasmic ends of S6 form the pore constriction that gates on depolarization. The transmembrane segments S1–S4 of each domain assemble as the voltage-sensor units. S4 has a characteristic array of positively charged residues of arginine and lysine, which are involved in sensing the transmembrane voltage. *Chlamydomonas* CAV2 shares these general features of the voltage-dependent calcium channels: CAV2 also has five or six arginine residues in each S4 helix and has EEEE motif, which are requisite for voltage sensing and Ca^{2+} selectivity. The carboxyl-terminal region consists of an IQ motif, which possibly binds to Ca^{2+}/calmodulin.

The 24 transmembrane segment structure of calcium channels has been believed to be generated by two successive internal duplications of a gene encoding six transmembrane segment protein. Various types of calcium channels have arisen during the course of evolution. High-voltage-activated channels (HVA) consist of Ca_v1 channels (also called L-type channels) and Ca_v2 channel (N-, P/Q-, and R-types). Low-voltage-activated channels (LVA), or Ca_v3 channel (T-type), are activated by weaker depolarization. These types are clearly distinct in vertebrates with respect to the amino acid sequence, channel kinetics, and pharmacology. The genome of fruit fly also has these types of calcium channels. By contrast, phylogenetic analysis shows that *Chlamydomonas* CAV2 positions at the root of the above vertebrate types and does not display specific homology with any of the channel types. The CAV2 sequence thus indicates that the distincttypes of the voltage-dependent calcium channels diverged after the evolution of multicellular organisms, and CAV2 represents the prototype of eukaryotic voltage-dependent calcium channels.

Closer examination, however, suggests that CAV2 has some features specific to the channel types of 1. Flagellar response on direct electrical stimulation of a single cell captured on a suction electrode demonstrated that the flagellar response is blocked by verapamil and diltiazem but not by ω-conotoxin GVIA (Yoshimura et al. 1997). Because verapamil and diltiazem generally block Ca_v1 and ω-conotoxin GVIA inhibits Ca_v2, CAV2 probably have pharmacological characteristics of Ca_v1.

Ca^{2+} is responsible for the control of various intracellular processes, but excessive exposure to Ca^{2+} is toxic to cells. Therefore, the influx of Ca^{2+} needs to be regulated precisely to eliminate the time and the position of Ca^{2+} concentration increase (see for review, Minor and Findeisen 2010). HVA detects the increase in Ca^{2+} concentration by calmodulin (CaM), which binds to the IQ motif in the C-terminal stretch. CAV2 is probably regulated in a similar manner because CAV2 also has IQ motif in the C-terminal stretch. The IQ motif of CAV2 resembles to that of Ca_v2 rather than Ca_v1 in that the residue at +5 position relative to the central isoleucine of IQ motif is hydrophilic as in Ca_v2, whereas Ca_v1 has a conserved

aromatic residue at this position. Ca_v1 interacts with CaM at six aromatic residues in the IQ motif, but CAV2 has only two aromatic residues.

HVA binds to β subunit, a soluble cytoplasmic polypeptide, at the conserved AID (α interaction domain) motif that is present in the loop connecting the sixth transmembrane segment of the domain I (IS6) and the first transmembrane segment of the domain II (IIS1). β subunit is essential to the channel activity. CAV2, however, does not have this AID motif and thus does not seem to interact with conserved β subunit. The IS6-IIS1 connector of CAV2 is possible to interact with alternative cytoplasmic proteins because a polypeptide co-precipitates with the IS6-IIS1 loop (Fujiu and Yoshimura, unpublished data).

Taken together, CAV2 shows several characteristics similar to HVA calcium channels with respect to the presence of IQ motif, association of subunit at the IS6–IIS1 loop, and pharmacology. High threshold of HVA is consistent with the phenotype of *ppr2*, which is defective in generating action potential by large depolarization.

5 Mechanosensitive Channels of *Chlamydomonas*

Mechanical stimulation excites membrane potential by activating mechanosensitive channels. The activities of mechanosensitive channels have been detected both in the flagella and the cell body of *Chlamydomonas*. Application of mechanical stimuli by applying a suction to *Chlamydomonas* flagella by way of glass micropipette evokes repetitive Ca^{2+} currents at a frequency of 0.5–1.0 Hz (Yoshimura 1996). The mechanoresponse is inhibited by Gd^{3+}, a blocker for mechanosensitive channels, and by verapamil, indicating that the current is triggered by mechanosensitive channels and amplified by voltage-dependent calcium channels. Application of a suction to the cell body membrane also activates mechanosensitive channels that pass Ca^{2+} and Ba^{2+} (Yoshimura 1998).

On avoiding reaction, the collision to obstacles is likely to be detected by the mechanoreceptor present at the flagella because flagella are the only part outside the cell body, which is covered by rigid cell wall. The molecular entity of the flagellar mechanoreceptor is TRP11, an ortholog of transient receptor potential (TRP) channels (Fujiu et al. 2011). TRP channels are a family of multimodal channels that respond to mechanical stimuli, temperature, and chemicals (Damann et al. 2008). TRP channels have been grouped into TRPA, TRPC, TRPM, TRPP, and TRPV subfamily channels based on the amino acid sequence and the sensitivity to agonists. TRP11 is included in TRPV family channel, which is named after the sensitivity to vanilloids such as capsaicin. The transmembrane domains of TRP11 display homology to *Drosophila melanogaster* nan and *Caenorhabditis elegans* OSM-9, TRPV channels that mediate mechanoreception in ciliated neurons. Although TRPV channels can be further separated into subfamilies (TRPV1, TRPV2, TRPV3, TRPV4, TRPV5, and TRPV6) that are activated at different temperature, chemicals, and mechanical stress, TRP11 does not display specific

similarity to any subfamilies suggesting that TRP11 represents a prototype of TRPV channels.

Importantly, TRP11 is responsible for the mechanoreception in motile flagella. Eukaryotic cilia and flagella have been thought to be split into separate groups of motile (but not sensory) cilia/flagella and sensory (but not motile) cilia/flagella. Therefore, *Chlamydomonas* flagella represent a novel type of cilia/flagella that are both motile and sensory. It is possible that *Chlamydomonas* flagella retain the ancestral form of eukaryotic cilia and flagella, i.e., a form before segregation into motile ones and sensory ones.

Besides the mechanosensitive channels that are aimed to detect collisions, some mechanosensitive channels are present in the intracellular membranes. *Chlamydomonas* cells express MSC1, a member of MscS-like protein, in the membranes around nucleus and within chloroplasts (Nakayama et al. 2007). MscS (mechanosensitive channels of small conductance) is a mechanosensitive channel that has been ubiquitously found in prokaryotes, and MscS-like proteins have been found in some cell-walled organisms of algae, land plants, and fungi such as *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, and *Schizosaccharomyces pombe* (Haswell 2007). MSC1 displays mechanosensitive gating when expressed and analyzed in *E. coli* cells. MSC1 is selective for monovalent anions as permeable ions, whereas *E. coli* MscS is almost non-selective. On application of ascending and descending ramps of mechanical stimuli, MSC1 opens at large stimulus intensity during ascending ramp but closes at small stimulus intensity during descending ramp indicating that hysteresis is present in the gating kinetics of MSC1. The suppression of MSC1 expression by RNAi results in an abnormal localization of chlorophyll suggesting that MSC1 is involved in the organization of chloroplast membranes. Preliminary analysis indicated that another MscS-like protein of *Chlamydomonas*, MSC3, is also expressed in the intracellular membranes (Nakayama and Yoshimura unpublished). Given that *Arabidopsis* MscS-like proteins are also expressed in the intracellular membrane and regulate the shape of plastids (Haswell and Meyerowitz 2006, Haswell et al. 2008), eukaryotic MscS-like proteins are likely to have functions of detecting and organizing the shape of intracellular membranous structures.

6 Flagellar Localization of *Chlamydomonas* Channels

Three genes have been identified as flagellar ion channels in *Chlamydomonas* so far: the voltage-dependent calcium channel CAV2, mechanosensitive channel TRP11, and PKD2, which is involved in mating (Huang et al. 2007). Flagella probably have still more channels: as mentioned above, voltage-dependent calcium channels that are activated by small depolarization and are reguired for phototaxis are likely to be present; The channels that are activated by intracellular acidification and induce Ca^{2+}-dependent deflagellation are probably also present (Quarmby

1996); TRP channels whose functions are yet to be explored have been detected in flagella (Fujiu et al. 2011).

It is noteworthy that phototaxis, photophobic response, mechanoreception, and flagellar excision are all brought about by the Ca^{2+} influx at flagella. The spatial and temporal control of the Ca^{2+} influx and subseguent elevation of intracellular Ca^{2+} concentration is thus important for coordination of these responses. It should be noted that the conversion of flagellar beating modes and the flagellar excision occur at the same Ca^{2+} concentration ($>10^{-6}$ M) and therefore the Ca^{2+} concentration at the flagellar base should not increase above threshold level during the reversal of swimming direction. Indeed, CAV2 is expressed exclusively in the mid-to-distal region of flagella (Fujiu et al. 2009) (Fig. 2b). Avoiding the expression at the proximal region probably prevents untimely activation of Ca^{2+}-dependent deflagellation machinery present at the base of flagella. In contrast, TRP11 is expressed mainly in the proximal region (Fujiu et al. 2011) (Fig. 2b). Targeting the mechanosensor to the proximal region probably eliminates the influence of flagellar motility and avoids false activation by the flagellar bending motion.

The turnover of flagellar components occurs steadily even though the length of flagella is constant (Watanabe et al. 2004). The components are brought toward the flagellar tip by the anterograde intraflagellar transport (IFT) and in the opposite direction by the retrograde IFT, which take place in the space between axoneme and flagellar membrane (Kozminski et al. 1993). The "cargo" to be transported is pre-assembled in the cell body as IFT particles and loaded on the IFT system. Because elimination of IFT abolishes the flagellar localization of CAV2 and TRP11, it is likely that CAV2 and TRP11 are also transported to the flagella by IFT (Fujiu et al. 2009, 2011). PKD2, which is expressed evenly along the entire length of flagella, is also transported by IFT (Huang et al. 2007). Therefore, flagellar channel proteins seem to be transported to the target sites by IFT irrespective the pattern of localization.

7 Future Perspectives

The present chapter has outlined the cellular and molecular mechanisms for the membrane excitation in *Chlamydomonas*. With the help of various molecular tools developed for *Chlamydomonas*, several genes encoding ion channels have been identified: a flagellar voltage-dependent calcium channel (CAV2), a flagellar mechanoreceptor protein (TRP11), and an intracellular mechanosensitive channel (MSC1). Nonetheless, a lot remain to be elucidated. Of special interest are (1) the flagellar calcium channel reguired for phototaxis, (2) the physiological role of MscS-like proteins in the intracellular membrane, (3) the functions of various TRP channels expressed in flagella, (4) the mechanisms of proximal and distal localization of CAV2 and TRP11, and (5) the molecular entity of the acid-activated Ca^{2+}-permeable channel for deflagellation. Future studies on these issues are expected to provide basic understanding of the Ca^{2+} signaling in *Chlamydomonas*.

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Cyclic Nucleotide Gated Channels (CNGCs) and the Generation of Ca^{2+} Signals

Wei Ma and Gerald A. Berkowitz

1 Introduction

Ion channels are intrinsic membrane proteins that facilitate ion diffusion across membranes. In this context they can be thought of as rather remarkable enzymes with the fastest known catalytic activity. Conductance rate through the pore of channel proteins (~10^8 ions/s) can approach that of the free ion diffusion potential in an aqueous solution. At the same time, channels are *gated*; permeation through their pore can only occur when factors that regulate this conductance lead to physical changes in the protein structure that allow for ion movement through the conduction pathway of the protein.

Ion channels facilitate passive movement of an ion; flux of ions across membranes occurs through ion channel proteins in response to the membrane electrochemical gradient (for that ion). The cytosol of plant cells typically has a negative membrane potential with respect to the apoplast outside of a plant cell (~150 mV) and the vacuole (<50 mV) (Bates et al. 1982; Jeworutzki et al. 2010); thus favoring the passive movement of cations into the cytosol. In the specific case of Ca^{2+}, concentration gradients (as well as the negative membrane potential of the cytosol with respect to the apoplast and vacuole) also provide a strong driving force for the passive movement of this cation into the cytosol of plant cells. Estimates of [Ca^{2+}] in the cell wall and vacuole are 1–10 mM while the cytosolic [Ca^{2+}] is tightly controlled and maintained at 100–200 nM and well below 1 μM even during signaling events (Mariani et al. 2003; Lecourieux et al. 2006). Thus, in the case of Ca^{2+}, the

W. Ma
Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA

G.A. Berkowitz (✉)
Agricultural Biotechnology Laboratory, Department of Plant Science, University of Connecticut, 1390 Storrs Road, Storrs, CT 06269-4163, USA
e-mail: gerald.berkowitz@uconn.edu

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electrochemical gradient between the cytosol and the two major pools of Ca^{2+} available for the generation of Ca^{2+} signals in cells would strongly favor passive movement of Ca^{2+} into the cytosol through ion channels that are gated open.

These two properties of channels, their high "enzymatic activity" and their gating properties, should, in theory, then dominate the generation as well as the properties of a Ca^{2+} signal occurring within a cell. In other words, when channels in a cell membrane are open, the conductance they facilitate should alter ion concentration to an extent more than other types of ion-conducting proteins, such as ATPases and antiporters. Ion channels as well as these other proteins are known to facilitate movement of Ca^{2+} across plant cell plasma (cell delimiting) and/or intracellular membranes. Thus, they can all be thought of as potentially contributing to the generation and shaping of a Ca^{2+} signal in a plant cell. In the simplest case, the Ca^{2+} signal can be presumed to be a temporary elevation in cytosolic [Ca^{2+}]. However, intracellular organelles such as chloroplasts, mitochondria, and the nucleus also are capable of generating Ca^{2+} signals specific to these compartments (Xiong et al. 2006).

Factors that lead to the opening of ion channels should provide a basis for the generation of a Ca^{2+} signal. Movement through open channels should dominate the net flux into the cytosol as influenced by Ca^{2+} efflux occurring through ATPases and antiporters. This paradigm has been posited for Ca^{2+} signaling in animal cells (Taylor et al. 2009) and is likely also true for Ca^{2+} signaling in plant cells. Thus, elucidation of the nature of Ca^{2+} signaling in plants should include an analysis of the molecular properties of Ca^{2+} channel protein complexes. Unfortunately, at the present time, much remains unknown in this area. This chapter includes a review of recent work germane to the molecular architecture of a specific class of plant Ca^{2+}-conducting channels (and how their function impacts some specific Ca^{2+}-dependent signaling cascades). We also present some speculations about how the function and regulation of these channel proteins might underlie the "coding" and "decoding" of Ca^{2+} signals in plants. Unless otherwise noted, the work that is discussed refers to *Arabidopsis* plants, genes, and coding sequences.

2 Candidates for Ca^{2+}-Conducting Channels

Higher plants contain no canonical genes encoding (four domain-type) voltage-gated Ca^{2+} channels found in animal cells (Ward et al. 2009; Verret et al. 2010). Of the 57 genes encoding cation-conducting channels in the *Arabidopsis* genome, 20 are members of the cyclic nucleotide-gated channel (CNGC) family (Mäser et al. 2001; Kaplan et al. 2007). CNGCs are cation-conducting channels that are activated by binding of cyclic nucleotide to the cytosolic portion of the channel. Current reviews point to CNGCs as facilitating the inward conduction of Ca^{2+} into the cell that occurs during (at least some) Ca^{2+}-signaling in plants (Wheeler and Brownlee 2008; McAinsh and Pittman 2009; Dodd et al. 2010; Verret et al. 2010). Functional analysis of translation products of CNGC-coding sequences in heterologous

expression systems (Leng et al. 1999; Ali et al. 2006; Frietsch et al. 2007; Urquhart et al. 2007) and phenotype analysis associated with translational arrest of their expression in plants (Ma et al. 2006; Ali et al. 2007; Frietsch et al. 2007; Urquhart et al. 2007; Ma et al. 2009; Guo et al. 2010; Ma et al. 2010; Qi et al. 2010) provides evidence that some of the CNGCs form Ca^{2+}-conducting channels; these include CNGC 1, 2, 10, 11, 12, and 18. We also have evidence (R. Ali, G.A. Berkowitz unpublished data) that CNGC5 (expressed in yeast) conducts Ca^{2+}. In one case (CNGC3) similar studies suggest channels formed by this expression product do not conduct Ca^{2+} (Gobert et al. 2006). Although the evidence is not definitive in all cases, the work cited above suggests that at least some of the CNGC gene translation products are components of Ca^{2+}-conducting channels in plants. Monovalent cation conductance by animal CNGCs is blocked by Ca^{2+} (Biel 2009). Patch clamp analysis of a plant CNGC expressed in *Xenopus laevis* oocytes demonstrated a similar block (Leng et al. 2002). Thus, the millimolar levels of Ca^{2+} present in the plant cell wall might restrict K^+ permeation through native plant CNGCs.

3 Evidence for the Presence of Functional CNGCs in the Plant Cell Plasma Membrane

Patch clamp studies indicate that the major inward Ca^{2+} current across the plant plasma membrane occurs through nonselective weakly voltage-gated cation channels (Demidchik et al. 2002; Demidchik and Maathuis 2007). Most relevant experimental evidence indicates that plant CNGCs are specifically localized to the plasma membrane (Schuurink et al. 1998; Arazi et al. 1999, 2000; Balagué et al. 2003; Ali et al. 2006, 2007; Gobert et al. 2006; Borsics et al. 2007; Christopher et al. 2007; Frietsch et al. 2007; Baxter et al. 2008), although CNGC20 may be targeted to the chloroplast (Sherman and Fromm 2009). Some evidence indicates that CNGC16 may be present in the endoplasmic reticulum and CNGC 7 and 8 are targeted to the tonoplast (Chang et al. 2007). A recent study (Pottosin et al. 2009) using the micro-electrode ion flux estimation (MIFE) technique to monitor net flux of Ca^{2+} has found that application of cAMP (but not cGMP) increased net efflux of Ca^{2+} from isolated beet root vacuoles. This result is consistent with the possible presence of CNGCs in the tonoplast of root cells.

Application of cyclic nucleotides to leaf cell protoplasts activates an inwardly rectified Ca^{2+} current across the plasma membrane (Lemtiri-Chlieh and Berkowitz 2004). Application of cAMP (Ma et al. 2009) and cGMP (Qi et al. 2010) results in an elevation of cytosolic $[Ca^{2+}]$ in plant leaf mesophyll cells; translational arrest of CNGC2 reduces the generation of this Ca^{2+} signal. Work with isolated tobacco (*Nicotiana plumbaginofolia*) protoplasts has shown that cAMP and cGMP application leads to cytosolic Ca^{2+} elevation as well (Volotovski et al. 1998). The electrophysiological characterization of cyclic nucleotide-dependent Ca^{2+} conductance across the plasma membrane included studies showing ligand activation of the

current in the detached patch configuration (i.e., in the absence of endogenous cytosolic signaling molecules), suggesting that it is due to a direct interaction between ligand and channel (Lemtiri-Chlieh and Berkowitz 2004). Thus, this evidence documents the presence of native CNGC channels in the plant plasma membrane.

4 Ligand and/or Voltage Gating of Animal and Plant CNGCs

The plant CNGC family of proteins was originally categorized as such some years ago due to domain homology analysis; they are similar in overall polypeptide structure to animal CNGCs (Mäser et al. 2001; Talke et al. 2003). Animal CNGCs [six genes are present in mammals; four "A" subunits (CNGCA1-4) and two 'B' subunits (CNGCB1 and CNGC3 – there is no CNGCB2 in animals)] were identified as channels that bind cyclic nucleotide cAMP and/or cGMP and, upon ligand binding, are activated and conduct cations (monovalent and divalent ions with varying degrees of selectivity) (Kaupp and Seifert 2002). More recent reviews of animal channels identify two related groups of channels that conduct cations upon binding of cyclic nucleotides; cyclic nucleotide gated (CNGC), and hyperpolarization activated, cyclic nucleotide gated (HCN) channels (Craven and Zagotta 2006; Biel 2009; Biel and Michalakis 2009; Biel et al. 2009). Animal CNGC channels are not gated by membrane potential; only binding of ligand affects their conductance. Patch clamp analysis of animal CNGCs shows that their conductance at a specific voltage relative to their maximal conductance is defined by nearly a straight line (Craven and Zagotta 2006). HCNs (four genes are present in mammals) are primarily activated by hyperpolarizing membrane potentials, but binding of cyclic nucleotides increases the open probability of the population of channels present in a membrane at a given hyperpolarizing membrane potential. Animal HCN channels do not conduct Ca^{2+} to much of an extent relative to monovalent cations (Biel et al. 2009) while animal CNGC channels do (Biel and Michalakis 2009). The amino acid residues experimentally confirmed to form the ion selectivity filters found in plant CNGCs are different from those found in any other family of plant or animal channels, including the animal HCN and CNGC channels (Mäser et al. 2001; Hua et al. 2003b; Yuen and Christopher 2009).

Electrophysiological (voltage clamp) analysis of plant CNGC channel currents upon expression of coding sequences in heterologous systems as well as voltage clamp analyses of cyclic nucleotide activated Ca^{2+} current in native membranes (Leng et al. 1999, 2002; Hua et al. 2003b; Lemtiri-Chlieh and Berkowitz 2004) indicates that these channels are clearly voltage gated, and that application of cyclic nucleotide increases the open probability of the channels. These properties of plant CNGCs make them functionally more akin to animal HCNs than animal CNGCs. With regard to the voltage gating of plant CNGCs, they appear to be closed at membrane potentials less negative than ~ −60 mV, and perhaps fully open at potentials more negative than −120 MV (an estimation) in the presence of cyclic

nucleotide. These gating characteristics indicate that at the typical membrane potentials present between the apoplast and cytosol in plant cells, an increase in the cytosolic concentration of cyclic nucleotide (the activating ligand) would lead to inwardly rectified movement of Ca^{2+} into the cell through these channels. As noted above, prior studies indicate that the major pathway for Ca^{2+} uptake into plant cells occurs through voltage-gated nonselective channels. Thus, the voltage dependence of plant CNGCs, their activation (by cyclic nucleotide) at membrane potentials that are present across the plant plasma membrane, and their inward rectification are consistent with electrophysiological analysis of native Ca^{2+}-conducting channels present in the membranes of plant cells. Some current reviews of Ca^{2+} signaling in plants (e.g., McAinsh and Pittman 2009; Ward et al. 2009) distinguish CNGCs as distinct from the (unknown) family of genes responsible for hyperpolarization activated (inwardly rectified) Ca^{2+} currents across the plasma membrane. The aforementioned information about the voltage dependence of plant CNGC-dependent currents suggests that they may be incorrectly categorized as "solely" ligand-gated channels.

Another aspect of ion conduction through plant CNGCs germane to the generation of Ca^{2+} signals in plant cells that can be discerned from the voltage clamp analyses mentioned above regards their inactivation, or lack thereof. Animal voltage-gated Ca^{2+} channels undergo rapid inactivation (Dolphin 2009). Currents through plant CNGCs are non-inactivating (this is also the case with animal CNGC and HCN channels); when clamped to a hyperpolarizing membrane potential, their presence in a membrane facilitates inward current that remains maximal as long as the step voltage is maintained. Thus, once they are activated (by generation of a rise in cytosolic cyclic nucleotide, for example, during a signaling cascade), they would remain open and conduct Ca^{2+} into the cell (and this conductance would not necessarily directly depolarize the cell due to the low cytosolic $[Ca^{2+}]$ maintained even during a signaling event). Only when a condition is generated (for example, breakdown of cyclic nucleotide) that would lead to closure of these channels, would the activity of other proteins involved in Ca^{2+} transport significantly contribute to shaping a cytosolic Ca^{2+} signal. A rise in cytosolic Ca^{2+} during a signaling event would lead to increased activity of Ca^{2+}/H^{+} antiporter or exchanger (CAX) proteins and Ca^{2+}/calmodulin activated Ca^{2+}-ATPases; these Ca^{2+}-tranlocating proteins could transfer Ca^{2+} out of the cytosol into intracellular compartments or out of the cell (Dodd et al. 2010). The generation of an initial cytosolic Ca^{2+} rise through activation of channels such as CNGCs would then turn on the CAX and Ca^{2+}-ATPases efflux systems. However, the transient Ca^{2+} signal could only occur, even if the efflux machinery is activated, if the channel facilitating the initial cytosolic Ca^{2+} increase is closed due to some change in cytosolic conditions that would lead to channel closure. Current reviews (Dodd et al. 2010) suggest that the amplitude, shape, and oscillatory aspects of a Ca^{2+} signal may impart some specificity to the readout, or decoding of the signal in plant cells. So the cytosolic factors that cause plant CNGC channels to close could contribute significantly to the shaping of the signal.

4.1 CNGC Structure

Animal CNGC and HCN channels are tetrameric; this quaternary structure is similar to the "inverted tee pee" found in members of the superfamily of six-transmembrane (TM1-6) "Shaker-like" pore-loop ion channels (Biel 2009). Each of the four subunits forming the ion conduction pathway through the membrane has a pore region selectivity filter (between TM5 and 6) that determines the selectivity of ion permeation. The carboxyl- and amino-termini of each polypeptide also resides in the cytosol. The cytosolic region of the carboxyl end of plant CNGC polypeptides has a cyclic nucleotide-binding domain and an overlapping calmodulin-binding domain.

In animals, native CNGCs are uniformly heterotetramers formed by two and often three different CNGC subunits (Biel 2009; Biel and Michalakis 2009). This is probably the case with animal HCN channels as well (Biel et al. 2009). No experimental evidence supports this quaternary structure in plant membranes. However, modeling studies suggest they are tetramers (Hua et al. 2003b). Expression of cDNAs encoding single plant CNGC gene products in heterologous systems such as oocytes and cultured human embryonic kidney cells, along with patch clamp analysis of currents, indicates that functional channels can be formed as homomeric protein complexes. However, it is unclear at present whether or not native plant CNGC channels are homo- or heterotetramers. Indirect evidence (Jurkowski et al. 2004; Yoshioka et al. 2006; Qi et al. 2010) suggests that plant CNGCs involved in Ca^{2+} signaling cascades may be heterotetramers.

CNGCs are the only plant channels that are activated (i.e., showing increased open probability at a given membrane potential) by cyclic nucleotides. They have cyclic nucleotide-binding domains (CNBDs) at the cytosol-localized carboxyl terminus. Several tertiary three-dimensional structural models of the plant CNGC CNBD have been generated (Hua et al. 2003a; Bridges et al. 2005; Kaplan et al. 2007; Baxter et al. 2008), and these studies identified residues that may contribute to ligand binding. Members of the plant "Shaker-like" (i.e., 6 transmembrane) K^+-selective KAT and AKT channel families also have carboxyl terminus CNBD domains, however, in these cases cyclic nucleotide deactivates the channel by shifting the voltage threshold for activation to more negative values (e.g., Hoshi 1995). At present, it is thought that plants lack functional cyclic nucleotide-activated protein kinases (Bridges et al. 2005; Kaplan et al. 2007; Martinez-Atienza et al. 2007). Therefore, CNGCs may be a primary cellular target of cyclic nucleotides that can transduce elevation of these messenger molecules in the cytosol to downstream steps of a signaling cascade in plants. It should be noted, however, that the monovalent cation/proton antiporter NHX7/SOS1 also has a CNBD of unknown function (Maathuis 2006). It is intriguing to note also that SOS1 expression is related to expression of CNGCs as well (Oh et al. 2009).

Most of the functional characterizations of cyclic nucleotide effects on plant CNGCs have shown that cAMP acts to activate the channel. Only in a few instances has cGMP been used as an activating ligand (Leng et al. 1999). Our understanding

of cyclic nucleotide signaling in plants that involves CNGCs, therefore, is primarily based on studies using cAMP.

4.2 Heterometric Assembly of CNGCs

As mentioned above, native animal CNGCs are assembled as heterotetramers comprised of translation products from several different CNGC genes. A region of some CNGC polypeptides near the carboxyl-terminus; a carboxyl-terminal leucine zipper (CLZ) domain (Zhong et al. 2002, 2003) is essential for the protein–protein interactions that lead to the assembly of the tetrameric animal CNGC protein. Further, this CLZ domain leads to preferential assembly of heterotetramers (rather than homotetramers) comprised of translation products of different CNGC genes. The CLZ domain in animal CNGCs is downstream (i.e., toward the carboxyl terminus) from the CNBD, and is in a region of the polypeptide that extends from the membrane into the cytosol. The CLZ domain of animal CNGCs contains regularly spaced, conserved leucine and/or hydrophobic residues. This conserved CLZ sequence is shown in Fig. 1a for four animal CNGC A polypeptides; the conserved leucine/hydrophobic residues in the CLZ domain of the animal CNGCs are identified in bold (red) type, and of these leucine/hydrophobic residues, the ones that facilitate protein–protein interactions between CNGC subunits are highlighted with a "*" in Fig. 1a.

At present, a similar domain has not been identified in plant CNGCs. ClustalW analysis was used to generate a comparison of the CLZ domain identified in an animal CNGC (the human CNGC A3 or hCNGCA3 polypeptide) to a similar region of *Arabidopsis* CNGC2 and 4. This comparison is portrayed in Fig. 1b. Some of the leucines and other hydrophobic residues present in this region of *Arabidopsis* CNGC2 and 4 might correspond to these conserved residues in the animal CNGC CLZ domain. This region of the *Arabidopsis* CNGC2 and CNGC4 polypeptides has residues (identified with a "*" in Fig. 1b) that might correspond to four of the six total conserved leucine/hydrophobic residues that facilitate protein–protein interactions in animal CNGCA polypeptides. Three leucine residues in the hCNGCA3 CLZ domain that are critical for heteromeric channel assembly (Zhong et al. 2003) are highlighted by arrows in Fig. 1b. Of these three residues, two may have corresponding residues in this region of *Arabidopsis* CNGC2 and 4. Thus, this speculative analysis suggests that this region of plant CNGCs might correspond to the CLZ domain present in animal CNGCA polypeptides, and may facilitate heteromeric assembly of plant CNGC protein complexes from the translation products of multiple CNGC genes.

Plant *cngc2* and *cngc4* null mutants have similar, but not identical, phenotypes (see below, regarding their involvement in pathogen defense responses) (Jurkowski et al. 2004). CNGC2 and 4 have overlapping expression patterns. Both mutants are modestly dwarfed and have altered pathogen defense responses. However, double *cngc2* and *cngc4* null mutants are extremely dwarfed, grow very poorly, and are

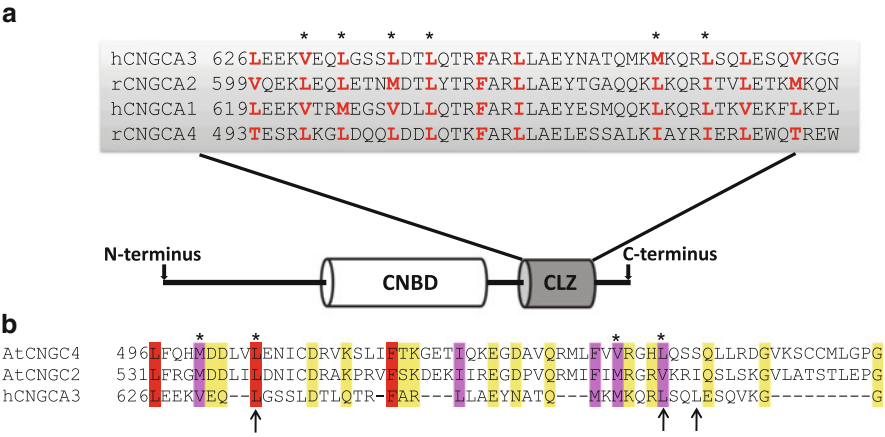

Fig. 1 (**a**) CLZ domains found in animal CNGCA polypeptides. Mammalian [human (h) or rat (r)] CLZ domain sequence alignment and the CNBD/CLZ cartoon were adapted from Zhong et al. (2003) and Zhong et al. (2002). The conserved hydrophobic residues are highlighted in *red* color and *bold* type. *Asterisks* denote those residues which were found to be crucial for hCNGCA3 CLZ domain interaction as described by Zhong et al. (2002). The position of the amino terminal residue for each polypeptide in (**a**) and (**b**) is indicated to the *left* of the sequence. (**b**) Multiple amino acid sequence alignment of the hCNGCA3 CLZ domain with two *Arabidopsis* (At) CNGCs (AtCNGC2 and 4). A region of the plant CNGC polypeptides corresponding to the CNBD and the remaining residues of the caryboxyl terminus was selected for the alignment. The ClustalW program was used to generate the alignment. As per the model shown in (**a**), the leucine/hydrophobic residues identified in the hCNGCA3 CLZ domain that are conserved among animal CNGCA polypeptides are highlighted in (**b**) by *red* shading when corresponding residues in AtCNGC2 and AtCNGC4 are identical, and with *purple* shading when the AtCNGC2 and AtCNGC4 residues are conservative substitutions. *Yellow* shading highlights other residues which are identical or show high similarity. *Asterisks* in (**b**) denote amino acids which are essential for hCNGCA CLZ interaction and display high similarity between hCNGCA3, AtCNGC2 and AtCNGC4. *Arrows* indicate three leucine residues (L633, L662, and L665; from *left* to *right*) found to be critical for heteromeric assembly of hCNGCA; AtCNGC2 and AtCNGC4 have identical or similar residues in two of these three positions. A *dash* denotes that there is no conservation of the amino acid residue

infertile (Jurkowski et al. 2004). These phenotypes are consistent with the possibility that (plant) CNGC2 and 4 polypeptides together form a channel protein in native membranes. A loss of either polypeptide could impair the same native channel protein complex. Loss of function of one of the two genes might lead to formation of a channel with incorrect subunit stoichiometry (i.e., it may be missing one of the polypeptides comprising the native channel) and is assembled into a partially functional tetramer without all the native polypeptides. Loss of function of both genes might prevent formation of any functional channel protein. A similar situation may be the case for CNGC11 and 12; the double null mutants are extremely dwarfed, infertile, and grow very poorly (K. Yoshioka, personal communication; also see Yoshioka et al. 2006).

4.3 Cytosolic Calmodulin Binds to and Inhibits Plant CNGC Function

Some experimental evidence has shown that the Ca^{2+}-binding protein calmodulin (CaM) binds to, and blocks, plant CNGC ion conductance (Köhler and Neuhaus 2000; Hua et al. 2003a; Li et al. 2005; Ali et al. 2006). As noted above, the CaM and cyclic nucleotide-binding domains on a plant CNGC polypeptide overlap. CaM may compete with cyclic nucleotide for binding to the channel, and therefore prevent cyclic nucleotide activation (Hua et al. 2003a). This, possibly competitive, interaction between cyclic nucleotide activation and CaM inhibition may have some ramifications for CNGC-dependent Ca^{2+} signaling. When increases in cytosolic Ca^{2+} due to cAMP addition and channel activation are prevented by chelation of Ca^{2+} with EGTA, application of CaM to the cytosol no longer blocks cAMP activation of the channel in studies of function using heterologous expression systems (Hua et al. 2003a). During some Ca^{2+} signaling events involving CNGCs (plant response to pathogens), the application of a CaM antagonist results in a greater cytosolic Ca^{2+} elevation (Ma et al. 2008). This result is consistent with the possibility that CaM may negatively affect CNGC conductance during signaling cascades in planta.

These experimental results are consistent with the following model. As cytosolic Ca^{2+} elevation occurs during a Ca^{2+}-signaling event, the Ca^{2+}/CaM complex formed may compete with cyclic nucleotide and reduce channel activation. A rise in cytosolic Ca^{2+} could lead to both channel closure and activation of efflux systems. This could shape the Ca^{2+} signal generated from a specific stimulus as a transient elevation and a rapid return to homeostasis. Of course, cyclic nucleotide phosphodiesterases (presumably) present in the plant cell cytosol could rapidly break down these secondary messengers after a signaling event. This removal of the CNGC activating ligand could also lead to transient Ca^{2+} elevations during signaling events involving CNGCs. Another important issue impacting CNGC-dependent Ca^{2+}-signaling events is the presence of Ca^{2+}-activated outwardly conducting anion (Cl^- permeable) channels in the plant cell plasma membrane. Cytosolic Ca^{2+} elevation during signaling events can lead to the activation of channels that facilitate outward Cl^- current which can depolarize the plasma membrane by 100 mV or more (Jeworutzki et al. 2010). So, even though Ca^{2+} influx might not directly depolarize the plasma membrane, the indirect effect of a Ca^{2+} signal on the plasma membrane membrane potential (through the activation of Cl^- efflux) could effectively close CNGCs; as mentioned above, they are voltage-gated channels.

5 Ca^{2+} Signaling, CNGCs, and Pathogen Defense Responses

CNGC-dependent Ca^{2+}-mediated plant defense responses to pathogens are a relatively well-characterized example of a Ca^{2+}-signaling cascade where involvement of specific Ca^{2+} channel genes is known. Plant immune signaling is triggered by

perception by plant cells of non-self through the recognition of pathogen-associated molecular pattern (PAMP) molecules and elicitor molecules that can be pathogen-derived toxins. Typically, PAMPs are evolutionarily conserved components of microbes (including pathogens) that are not present in the plant cell. Examples are lipopolysaccharide (LPS; the glycolipid component of the outer membrane found in Gram-negative bacteria), flagellin (the structural protein component of the bacterial motility organ), the bacterial elongation factor Tu, chitin (found in fungal cell walls) and ergosterol (found in fungal membranes) (Zipfel 2008).

One of the earliest components of the pathogen response signal transduction cascade in a plant cell is an increase in cellular Ca^{2+} (Nürnberger et al. 1994) upon perception of non-self (PAMP) presence. Cytosolic Ca^{2+} elevation upon pathogen perception leads to a suite of basal defense responses ("PAMP triggered innate immunity" as described by Jones and Dangl 2006), and the hypersensitive response (HR) to avirulent pathogens. HR, one of the immune responses triggered by specific effector molecules in pathogens, involves reactive oxygen species (ROS) and nitric oxide (NO) production leading to programmed cell death (PCD) in cells neighboring the infection site, which limits the spread of the disease (Bent and Mackey 2007). HR occurs when a specific avirulence (avr) gene product generated by pathogens interacts (directly or indirectly) with a corresponding resistance (or "*R*" gene encoded) protein present in the plant cell. PCD associated with HR that is evoked by this interaction is distinct from, and augments basal level innate immunity resistance responses. However, in both cases, a range of cytosolic defense systems is initiated in the cells at the infection site upon pathogen perception due to cytosolic Ca^{2+} elevation (Lecourieux et al. 2006; Ma and Berkowitz 2007). Pathogen recognition mechanisms lead to a cascade of defense responses through Ca^{2+} signaling (Aslam et al. 2008; Jeworutzki et al. 2010).

Early electrophysiological analysis of plant cell responses to pathogen perception demonstrated that plasma membrane Ca^{2+}-conducting channels contribute to pathogen-induced cytosolic Ca^{2+} elevation (Gelli and Blumwald 1997). Other early work (Grant et al. 2000) has shown that inoculation of leaves with a pathogen (*Pseudomonas syringae*) leads to an elevation of plant cell cytosolic Ca^{2+}. This Ca^{2+} elevation may be affected by the presence in the pathogen of some (but not all) avr genes. Exposure of leaves to a Ca^{2+} channel blocker prevents HR; this result supports the concept that pathogen/PAMP-associated influx of Ca^{2+} is an early signal initiating plant defense responses which, in the presence of pathogen avr and corresponding plant *R* genes, leads to HR. Mutations in several (*Arabidopsis*) CNGCs have been associated with altered plant responses to pathogens. Loss-of-function of CNGC2 (the "*d*efense-*no-d*eath" or *dnd1* mutant) and CNGC4 (the "*H*R-like *l*esion *m*imic *hlm1* or "*dnd2*" mutant) alters plant responses to avirulent pathogens (including *P. syringae*). *Arabidopsis* plants with these mutations display impaired HR, constitutive expression of salicylic acid (SA), altered expression of pathogen defense-related genes, and (despite the lack of HR) increased resistance to pathogen growth unrelated to the HR.

The *Arabidopsis* mutant "constitutive expresser of *PR* (pathogenesis related) genes 22" (*cpr22*) was identified in a screen for mutations associated with altered

activation of pathogen defense responses (Yoshioka et al. 2001). The *cpr22* mutant displays different phenotypes from *dnd1* and *hlm1* (*dnd2*) plants, but does overlap with these CNGC loss-of-function mutants in having constitutively activated defense responses and enhanced resistance to *P. syringae* (*cpr22* also was shown to have enhanced resistance to the oomycete pathogen *Hyaloperonospora parasitica*) (Yoshioka et al. 2001; 2006; Moeder and Yoshioka 2008;). The *cpr22* mutation was identified as a 3-kb deletion that fuses two CNGC genes, *CNGC11* and *CNGC12*, to generate a novel chimeric gene, *CNGC11/12* (Yoshioka et al. 2006). On the basis of genetic and molecular analyses, it is suggested that the phenotype conferred by *cpr22* is attributable to the expression of *CNGC11/12*. Analysis of the *CNGC11/12* mutant suggests that *CNGC11* and *CNGC12* forms heteromeric channel protein complexes that are positive regulators of plant resistance to avirulent pathogens (Yoshioka et al. 2006). Urquhart et al. (2007) further investigated the nature of HR-like cell death induced by expression of *CNGC11/12* using a transient expression system. In this study, cell death development was found to depend on Ca^{2+} influx into cells.

Recent work has correlated an increase in cAMP levels in plant leaves with the transient cytosolic Ca^{2+} spike occurring within minutes of pathogen perception (Ma et al. 2009). At a later time after pathogen inoculation (i.e., developing over several hours), cGMP levels rise (Meier et al. 2009). Other studies identify specific gene products as putative guanylyl cyclases that may play a role (i.e., activating CNGCs) in pathogen defense signaling. The translation product of the Wall-Associated Kinase-Like 10 gene has guanylyl cyclase activity in vitro and is expressed along with pathogen defense-related genes (Meier et al. 2010). Some evidence suggests that a leucine-rich repeat receptor-like kinase (AtPepR1) that binds endogenous peptide signals in *Arabidopsis*, and is involved in pathogen defense signaling may be a guanylyl cyclase and act upstream from CNGC2 in pathogen-induced cytosolic Ca^{2+} elevations (Qi et al. 2010; Krol et al. 2010). Inhibitors of nucleotidyl cyclases block pathogen-induced cAMP elevation and prevent the coincident Ca^{2+} elevation and downstream pathogen defense signaling (Ma et al. 2009). Inhibitors of cyclic nucleotide breakdown (i.e., cyclic nucleotide phosphodiesterase inhibitors) lead to a greater elevation in cAMP and a faster onset of pathogen defense responses, presumably due to a stronger Ca^{2+} signal (Ma et al. 2009). These pharmacological studies, along with the work mentioned above with plants that have mutations in CNGC genes and the study of cyclic nucleotide elevations during pathogen responses provide evidence that the Ca^{2+} signal generated during pathogen defense responses in plants is dependent on CNGC function. It should be noted, however, that there is some indirect evidence (Jeworutzki et al. 2010) that loss of function of CNGC2 (i.e., in the *dnd1* mutant) does not impact some pathogen defense signal transduction steps downstream from the initial Ca^{2+} signal; PAMP-induced membrane depolarization was found to be similar in wild type and *dnd1* leaf cells.

It remains unclear how the shaping of a Ca^{2+} signal occurring within minutes of pathogen perception can impact plant defense responses that may develop many hours or even days after the Ca^{2+} signal. However, recent work does support this notion; i.e., the shape of a Ca^{2+} signal can impact downstream events occurring

much later in a signal cascade. Zhu et al. (2010) found that translational arrest of an endomembrane-localized Ca^{2+}-ATPase resulted in an increased cytosolic Ca^{2+} signal and faster onset of HR in response to a pathogen elicitor in tobacco. Translational arrest of two tonoplast-localized Ca^{2+}-ATPases resulted in spontaneous PCD and other pathogen response-related phenotypes (Boursiac et al. 2010) suggesting a similar mechanism; blocking Ca^{2+} efflux from the cytosol increases a Ca^{2+} signal related to PCD and pathogen defense signaling. These studies are consistent with the results of work mentioned above with inhibitors of cyclic nucleotide breakdown and their effect of reducing the time for onset of HR.

As mentioned above, gating of plant CNGCs is known to be influenced by membrane potential, as well as the cytosolic secondary messengers cAMP and cGMP, and the Ca^{2+} sensor protein CaM. Conductance of ion channel proteins (in general) can also be influenced by other cytosolic factors. These post-translational protein modifications include phosphorylation/dephosphorylation (through the action of protein kinases and phosphatases), S-nitrosylation (by NO), and oxidation/reduction (for example, from H_2O_2 and other reactive oxygen species molecules). Some evidence suggests that protein phosphoryation, nitrosylation, and oxidation by H_2O_2 all may act in pathogen defense signaling cascades and impact Ca^{2+} signaling (Garcia-Brugger et al. 2006; Besson-Bard et al. 2008; Romero-Puertas et al. 2008). As noted above, plant CNGCs have been associated with Ca^{2+} signaling that occurs during pathogen defense responses. There is no direct evidence at present to link these post-translational modification systems to plant CNGCs; however, CNGCs could be the target of these systems.

Animal CNGCs can be activated by NO through a cysteine (C) residue S-nitrosylation (Broillet 2000). Broillet (2000) reported that residue C460 (between the sixth TM domain and the CNBD) of an animal CNGC is crucial for this NO sensitivity. Our analysis reveals that a cysteine residue (C466) of the plant CNGC2 polypeptide (also between the 6th TM domain and the CNBD) is surrounded by basic and acidic residues, which implies that this residue might be functioning as an S-nitrosylation site (S. Spoel, personal communication; also see Marino and Gladyshev 2010). However, CNGC2 does not have a classical conserved nitrosylation hydrophobic motif [(H/K/R) C (hydrophobic) X (D/E)] as suggested previously (Hess et al. 2005). In addition, a proteomic survey of proteins in *Arabidopsis* that are S-nitrosylated during pathogen defense responses did not identify any known channels.

6 CNGC Involvement in Other Ca^{2+}-Signaling Cascades

As mentioned above, CNGCs could be the primary targets of cyclic nucleotide signaling in plants. Cyclic nucleotide elevations during some Ca^{2+} signaling events and phenotypes induced by addition of exogenous cyclic nucleotide, then, provide indirect evidence for CNGC involvement in a number of other plant signaling cascades involving Ca^{2+} and/or Ca^{2+} signaling. CNGC expression patterns and

increases in expression during some signaling events provide evidence along the same lines. Recent reviews provide very extensive and excellent analyses of CNGC expression patterns and how some environmental cues impact CNGC expression (Kaplan et al. 2007; Yuen and Christopher 2009). Other reviews provide information about cyclic nucleotide signaling in plants (Martinez-Atienza et al. 2007; Meier et al. 2009; Gehring, 2010; Meier et al. 2010).

Movement of extracellular Ca^{2+} into the cytosol and resulting cytosolic Ca^{2+} elevation is a critical signaling event necessary for pollen tube elongation (Pierson et al. 1994; Malhó and Trewavas 1996). Application of exogenous cAMP has also been shown to positively affect pollen tube growth (Moutinho et al. 2001; Tsuruhara and Tezuka 2001) in *Zea mays* and *Lilium longiflorum*. The level of endogenous cAMP in pollen tubes is associated with differences in *Lilium longiflorum* pollen tube growth under cross-compatible and self-incompatible conditions (Tsuruhara and Tezuka 2001). CNGCs 7, 8, 16, and 18 are preferentially or specifically expressed in (*Arabidopsis*) pollen or growing pollen tubes (Bock et al. 2006). This body of work suggests an involvement of CNGCs in generating the cytosolic Ca^{2+} elevation that is an important signal in directed growth of the pollen tube during pollen germination. There is also direct evidence supporting this point. Recombinantly expressed CNGC18 increases the level of Ca^{2+} in *Escherichia coli*, and translational arrest of CNGC18 results in defective pollen tube growth (Frietsch et al. 2007).

Some evidence supports the involvement of cGMP in abiotic stress responses as well as some other physiological processes in plants. Donaldson et al. (2004) found a rapid (within 5 s) cGMP increase in *Arabidopsis* seedlings subjected to salinity (salt or osmotic treatments); application of a guanylyl cyclase inhibitor blocked the salt- and osmotic-stress induced cGMP elevation. Other work has shown that both salt and osmotic stresses can result in a Ca^{2+} elevation in the cytosol that is at least partially dependent on influx of Ca^{2+} into the cell across the plasma membrane (Knight et al. 1997; Tracy et al. 2008). Donaldson et al. (2004) noted that a salt-induced cytosolic Ca^{2+} elevation was reduced in the presence of the guanylyl cyclase inhibitor. However, in this study, the Ca^{2+} elevation induced by severe salinity stress could not be blocked by the inhibitor. This result suggests that intracellular stores of Ca^{2+} might contribute to the cytosolic Ca^{2+} elevation (as well as influx into the cell); this is consistent with other studies of salinity stress signaling (Tracy et al. 2008). The linkage of elevation of cytosolic cGMP and Ca^{2+} in salinity stress signaling suggests that CNGCs might be involved in this signal transduction pathway. Recent studies of CNGC19 and 20 in response to salinity stress (Kugler et al. 2009) indicate that this abiotic stress increases the expression of both genes (in various tissues); this work further supports the involvement of CNGCs in salinity stress responses.

Some studies identify cGMP, CNGC1, and CNGC10 involvement in root gravitropism (Hu et al. 2005; Ma et al. 2006; Borsics et al. 2007), a process known to involve Ca^{2+} signaling (Fasano et al. 2002; Blancaflor and Masson 2003). A CNGC has been identified in the barley aleurone layer (Schuurink et al. 1998). Gibberellic acid signaling in the cereal aleurone involves cGMP as well as cytosolic

Ca^{2+} and CaM (Gilroy 1996; Penson et al. 1996; Schuurink et al. 1996). Thus, CNGCs may be involved in signaling related to cereal aleurone function. We also find in recent work that Ca^{2+} conduction by CNGC2 functions during growth and development of plants to repress senescence signaling through NO generation (Ma et al. 2010).

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Annexins

Anuphon Laohavisit and Julia M. Davies

Abstract Annexins are small proteins capable of membrane attachment or insertion. Animal annexins can form Ca^{2+}-permeable conductances in planar lipid bilayers or vesicles. Some have also been implicated in the regulation of cytosolic free calcium. Similar results are now being reported for plant annexins, justifying their consideration as novel components of calcium-signalling networks. Peroxidation of bilayers alters the calcium transport characteristics of plant annexins, suggesting that they could act as intersections between calcium and reactive oxygen-signalling pathways. The ability of plant annexins to bind actin, hydrolyse ATP/GTP and act as peroxidases sets them apart from conventional calcium transporters. Here, their structure and functions are reviewed.

1 Introduction

Annexins are small, predominantly cytosolic proteins that appear to be expressed ubiquitously in eukaryotes and have now been detected in prokaryotes (reviewed by Gerke and Moss 2002; Hofmann 2004; Moss and Morgan 2004; Morgan et al. 2006). As Ca^{2+}-binding proteins, annexins have long been considered to operate as sensors of changes in free cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) or effectors in $[Ca^{2+}]_{cyt}$ signalling (Gerke and Moss 2002; Gerke et al. 2005). Increasing $[Ca^{2+}]_{cyt}$ can cause annexins to associate with the plasma membrane or endomembranes where they may form platforms for interactions with other proteins and exert regulatory effects. The ability of annexins to bind actin and modulate ion transport proteins could thus link local changes in $[Ca^{2+}]_{cyt}$ to cytoskeletal responses and propagative ionic events (reviewed by Gerke et al. 2005, Konopka-Postupolska 2007). It has also been proposed that the dissociation of annexins from membranes could

A. Laohavisit • J.M. Davies (✉)
Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK
e-mail: jmd32@cam.ac.uk

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contribute to $[Ca^{2+}]_{cyt}$ elevation in signalling, through the release of the Ca^{2+} ions that form the bridge between the protein and bilayer (Hoyal et al. 1996). A possible additional level of functional complexity comes from the observations that annexins can generate Ca^{2+}-permeable transport pathways (Liemann et al. 1996; Köhler et al. 1997; Langen et al. 1998; Rosengarth et al. 1998; Kubista et al. 1999; Bandorowicz-Pikula et al. 2003; Hegde et al. 2006; Kirilenko et al. 2006; Gorecka et al. 2007; Laohavisit et al. 2009) and so could function directly in the control of $[Ca^{2+}]_{cyt}$. Annexins thus stand in contrast to Ca^{2+}-binding proteins that affect a single type of mechanistic "output".

Eukaryotic cells seemingly express several annexins at any given point in the cell cycle and these may be recruited at different rates to several membranes (Babiychuk et al. 2009). Interpreting annexin function is further complicated by their having discrete enzymatic activities (such as peroxidase activity) that may vary with the level of association with Ca^{2+} and lipids (Gorecka et al. 2005; Mortimer et al. 2009). With such a diversity of location and possible function, coupled with potential redundancy in a given signalling pathway, it is perhaps unsurprising that precise roles in $[Ca^{2+}]_{cyt}$ signalling remain relatively poorly elucidated, even in animal cells. However, animal annexins are firmly implicated in the regulation of exo- and endocytosis, membrane repair and regulation of membrane composition (such as raft formation) (Liemann et al. 1996; Köhler et al. 1997; Langen et al. 1998; Rosengarth et al. 1998; Isas et al. 2000; Kourie and Wood 2000; Golczak et al. 2001; Bandorowicz-Pikula et al. 2003; Ladokhin and Haigler 2005; Hegde et al. 2006; Kirilenko et al. 2006). They are involved in normal cellular functions such as myocyte contraction but also operate in (a)biotic stress responses and apoptosis (Song et al. 2002; Wang et al. 2003; Monastyrskaya and Babiychuk 2009).

Plant annexins have been somewhat neglected as possible components of signalling or $[Ca^{2+}]_{cyt}$ homeostatic mechanisms. They form a distinct phylogenetic group and appear ubiquitous within the plant kingdom (Hofmann 2004; Moss and Morgan 2004; Morgan et al. 2006; Mortimer et al. 2008). Rice contains nine annexin genes while *Arabidopsis thaliana* contains eight, suggesting specific roles but also raising the spectre of redundancy for phenotypic studies of mutants. Estimates suggest that annexins comprise 0.1% of plant cell protein (Delmer and Potikha 1997) and proteomic studies suggest widespread membrane association (reviewed by Mortimer et al. 2008; Laohavisit and Davies 2009). Do they contribute to Ca^{2+} relations in plants? In vitro studies suggest that they have the potential (Hofmann et al. 2000a; Laohavisit et al. 2009). Here we examine how plant annexins might operate in Ca^{2+} relations, working through their structure to possible roles.

2 Ca^{2+} Binding and Lipid Binding by Plant Annexins

Plant annexins are typically in the range 32–36 kDa. Ca^{2+} binding is conferred by the annexin "repeat" of approximately 70 amino acids that contains the "endonexin" sequence (K-G-X-G-T-{38 variable residues}-D/E) and Type III

Ca^{2+}-binding site. (Gerke et al. 2005; Monastyrskaya and Babiychuk 2009; Laohavisit and Davies 2009). In plants only the first or fourth repeats are highly conserved (Hofmann 2004; Gerke et al. 2005; Fig. 1). A repeat forms five short

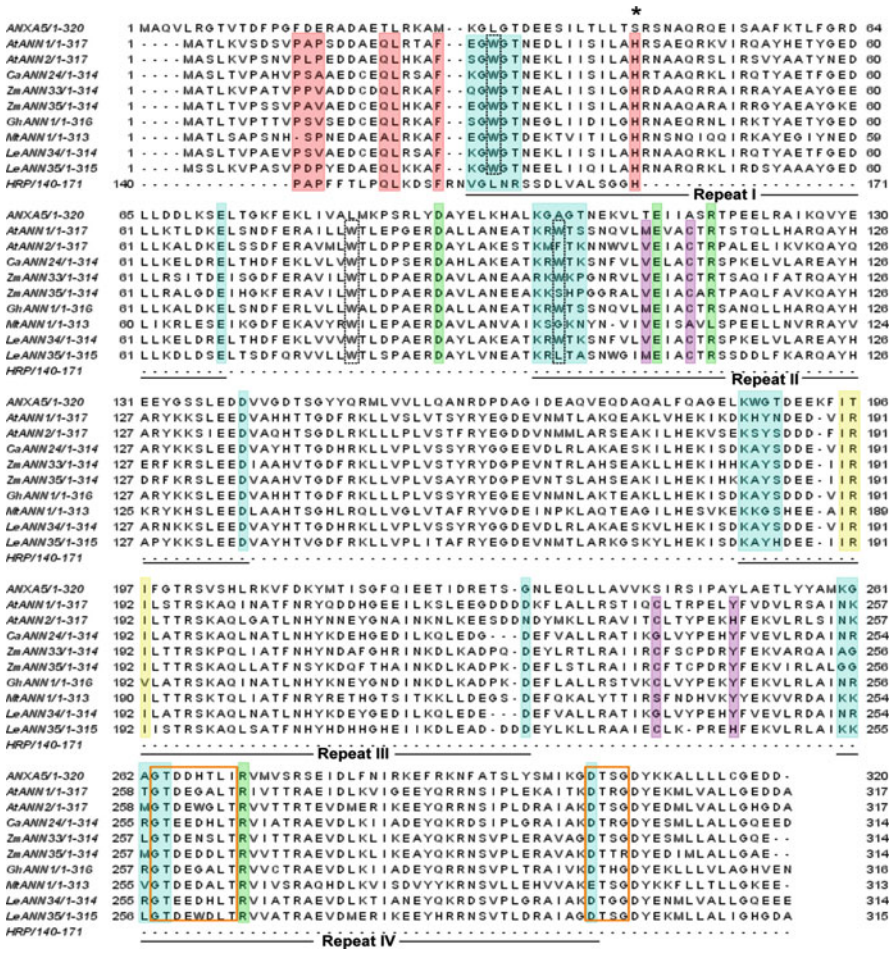

Fig. 1 Alignment of plant annexin amino acid sequences annexin A5 (ANXA5) and horseradish peroxidase (HRP). Annexin repeats (I–IV) are shown beneath the sequences. *Red*, haem-binding domain of peroxidase from *Armoracia rusticana* with the N-terminus of annexins with identical residues highlighted and the conserved His residue marked (*Asterisk*). *Purple*, putative S3 cluster (Hofmann et al. 2003) thought to be involved in redox reactions. *Green*, salt bridges involved in channel function of animal annexins (Liemann et al. 1996). *Yellow*, IRI motif for binding actin (Calvert et al. 1996). *Orange box*, putative GTP-binding motif (Clark et al. 2001). *Black dash box*, conserved tryptophan Ca^{2+}-independent membrane binding (Dabitz et al. 2005). *Teal*, Ca^{2+}-binding sequences. Alignment was performed using ClustalW and edited in JalView. Accession numbers are: *ANXA5* (gi:4502107); *AtANN1* (gb:NP174810); *AtANN2* (gb:201307); *CaANN24* (gb:CAA10210); *ZmANN33* (gb:CAA66900); *ZmANN35* (gb:CAA66901); *GhANN1* (gb:AAC33305); *MtANN1* (gi:22859608); *LeANN34* (gb:AAC97494); *LeANN35* (AAC97493); *A. rusticana* HRP (gb:CAA00083)

α helices, connected by loops that also contribute to Ca^{2+} binding. An annexin monomer is thought to form a curved disc with the convex side available for Ca^{2+} and bilayer binding. The number of Ca^{2+} ions that can be sequestered at the annexin–bilayer interface could therefore depend on the degree of endonexin conservation and degree of annexin oligomerisation.

At neutral pH, Ca^{2+} ions form a bridge between the annexin and negatively charged phospholipids of the bilayer. Although all annexins exhibit Ca^{2+}-dependent phospholipid binding, individual members differ in their requirement for Ca^{2+}, pH and phospholipid headgroup specificity. Different phospholipid headgroups include phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol, phosphatidic acid and phosphatidylcholine (Blackbourn et al. 1991; Balasubramanian et al. 2001). The mechanistic basis for lipid binding is better understood for animal annexins. For example, for vertebrate annexin A5 (ANXV) a PS-binding site is located in repeats I and II, overlapping with the Ca^{2+}-binding domain (Montaville et al. 2002). A glutamate residue (Glu72) binds both a Ca^{2+} ion and the serine ammonium group of PS. Individual annexin repeats have been shown to have different lipid specificities and are therefore not equivalent in the membrane-binding process. In vertebrate annexin A4, site-directed mutagenesis of the four annexin-conserved repeats revealed that repeat IV is able to accommodate the large headgroups of PS and PI whilst the other three repeats may form more restricted binding pockets (Sohma et al. 2001). In *Hydra* annexin B12, Ca^{2+}-dependent lipid binding is precisely tuned, rapid and highly co-operative (Patel et al. 2001). Once one Ca^{2+} ion binds, the resulting complementary spacing between the annexin and the lipid geometry might facilitate the additional binding of Ca^{2+}. The animal annexin N-terminus may also play a role in membrane interaction (Hofmann et al. 2000b).

Ca^{2+}-dependent binding to lipid bilayers is also a property of plant annexins (Smallwood et al. 1990; Blackbourn et al. 1991; Breton et al. 2000; Hofmann et al. 2000a; Hu et al. 2008). In vitro estimates show that micromolar Ca^{2+} may be required (Blackbourn et al. 1991). As for animals, the four repeats of plant annexins are not equivalent in membrane binding. It has been proposed that modules I/IV and II/III (comprising repeats I, IV and II, III, respectively) may act as individual membrane-binding units and different plant annexins may use either of these repeat pairs for membrane binding. Recently, the crystal structure of cotton annexin (GhANN1) has been resolved at 2.5 Å, demonstrating that module I/IV of the protein is responsible for phospholipid binding, using four Ca^{2+} to do so (Hu et al. 2008). Hydrophobic interactions are also involved. *Capsicum annum* CaANN24 attachment to membrane involves several amino acid residues hydrogen bonding to the phospholipid headgroup and glycerol backbone (Hofmann 2004). The N terminus (which is shorter than animal counterparts) may play an important role in membrane binding and confer specificity (Dabitz et al. 2005).

Membrane binding by some animal annexins marks a transition from soluble monomer to membrane-attached trimer (Isas et al. 2003). Trimer formation appears reliant on the core of the annexin rather than the terminal regions and may also be influenced by lipid composition of the membrane (Patel et al. 2005). The membrane-attached form is important for several functions including vesicle trafficking and

cytoskeleton attachment (Gerke and Moss 2002). The membrane-attached form is thought to be required for plant annexin activity including secretion and exocytosis (Carroll et al. 1998), response to low temperature (Breton et al. 2000), response to osmotic stress and ABA signalling (Lee et al. 2004). However, it is still unclear whether plant annexins also undergo oligomer formation on binding to the membrane despite Ca^{2+}-independent oligomerisation in solution (Hofmann et al. 2002).

3 Ca^{2+}-Independent Lipid Binding

Some animal annexins such as A5 and B12 also exhibit Ca^{2+}-independent membrane binding at acidic pH (Langen et al. 1998; Golczak et al. 2001; Isas et al. 2003; Patel et al. 2005). Acidic pH promotes a transmembrane form instead of the helix–loop–helix membrane-associated structure (Langen et al. 1998; Ladokhin et al. 2002; Isas et al. 2003; Patel et al. 2005). Protonation of negatively charged amino acids such as aspartic acid (Asp) or glutamic acid (Glu) at the hydrophobic face of the helix is implicated in this conformational change (Kim et al. 2005). The membrane-inserted form of annexin B12 is a monomer, unlike the trimeric membrane-attached form. The transitions between surface trimer and membrane-inserted monomer are reversible (Ladokhin and Haigler 2005). In addition to the membrane-attached form and the membrane-inserted form, annexin B12 can also exhibit a membrane peripheral form. At pH 5.5, it is peripheral (partially inserted) and parallel to the membrane. This form can be interconverted into the cytosolic form at pH 6.5 and to the transmembrane form at pH 4 (Hegde et al. 2006).

Plant annexins are also capable of Ca^{2+}-independent membrane binding, and this may occur at neutral pH (Blackbourn et al. 1991; Breton et al. 2000; Hofmann et al. 2000a, 2002; Dabitz et al. 2005). About 10–20% of the total annexin populations for both GhANN1 and CaANN32 are able to bind to lipid vesicles in the absence of Ca^{2+} at neutral pH. The basis of Ca^{2+}-independent binding was partly due to the three conserved tryptophan residues (W35, W88, W107) which are exposed on the convex side of the proteins as well as the basic residues such as Lys190 and Arg262 and 263, since replacing these residues affected the membrane-binding ability of CaANN24 (Dabitz et al. 2005; Fig. 1). Such Ca^{2+}-independent lipid-binding at neutral pH implies that plant annexins can attach to membranes independently of a $[Ca^{2+}]_{cyt}$ stimulus or stress-induced cytosolic acidification and may have roles to play at the membrane under "normal" conditions.

4 Oligomerisation and Post-translational Modification

Levels of homo- and hetero-dimerisations and oligomerisation are likely to affect annexin function in the soluble phase and when associated with the membrane phase. As noted previously, single-plant annexins can undergo Ca^{2+}-independent

oligomerisation (Hofmann et al. 2002). This can also be promoted by hydrogen peroxide (Gorecka et al. 2005; Konopka-Postupolska et al. 2009; Mortimer et al. 2009). As yet, hetero-oligomerisation by plant annexins remains unreported, but such capacity would expand the complexity of their operation further.

Annexins isolated from native tissue tend to have greater enzymatic activity (peroxidase) than those purified from heterologous expression systems (Gidrol et al. 1996; Gorecka et al. 2005; Laohavisit et al. 2009). This may indicate a degree of post-translational modification in native tissue. To date, plant annexins have been found to be capable of being phosphorylated (Andrawis et al. 1993; Gorecka et al. 2005; Agrawal and Thelen 2006), *S*-nitrosylated (Lindermayr et al. 2005) and *S*-glutathionylated (Konopka-Postupolska et al. 2009). *S*-nitrosylation of the cytseine residues of *Arabidopsis thaliana* annexin 1 (AtANN1) indicates its presence downstream of nitric oxide production in signalling but this has not yet been demonstrated directly. *S*-glutathionylation of AtANN1 (again at the cysteine residues) lies downstream of ABA and prevents the protein's irreversible oxidation, perhaps permitting its continued operation in signalling (Konopka-Postupolska et al. 2009). This modification also reduces AtANN1's Ca^{2+} affinity and so may determine its localisation as well as function (Konopka-Postupolska et al. 2009).

5 Annexin Action at Multiple Sites

Although resident in the cytosol, plant annexins have been identified in other aqueous compartments including the chloroplast stroma (Rudella et al. 2006) and phloem sap (Giavalisco et al. 2006). Although lacking the N-terminal signal peptide for secretion, two *Arabidopsis* annexins (AtANN1 and AtANN2) are predicted to be extracellular (Laohavisit et al. 2009) and have been isolated from cell walls (Kwon et al. 2005; Bayer et al. 2006). This raises the possibility that they may act at the plasma membrane from its extracellular face. As soluble proteins that could feasibly attach to a membrane during the latter's purification, some caution is needed in interpreting results from proteomic studies that are not augmented by in situ localisation of an annexin. Nevertheless, plant annexins have been identified in association with both plasma- and endo-membranes including tonoplast (Seals and Randall 1997; Carter et al. 2004), chloroplast envelope (Seigneurin-Berny et al. 2000), thylakoid (Friso et al. 2004) and nuclear membrane (de Carvalho-Niebel et al. 1998). The most abundant *Arabidopsis* annexin, AtANN1, appears capable of a plasma membrane association, plus tonoplast and thylakoid (e.g., Santoni et al. 1998; Carter et al. 2004; Friso et al. 2004; Laohavisit and Davies 2009). Immunofluorescence indicates the association of a *Medicago sativa* annexin (MsANN2) with the nucleolus, but no nuclear targeting sequences have been identified (Kovács et al. 1998). Sites of membrane association may largely depend on highly local conditions of lipid identity, $[Ca^{2+}]$, pH plus the presence of protein partners and nature of the annexin's post-translational modification.

The level of a given membrane association, attached, peripheral or integral, is poorly understood for plant annexins. There is evidence that they may exist in the membrane-inserted form (Santoni et al. 1998; Breton et al. 2000; Gorecka et al. 2007 and reviewed by Mortimer et al. 2008). For example, the 34-kDa isoform of AtANN1 associated with leaf plasma membrane is insensitive to the Ca^{2+} chelator EDTA (ethylene diamine tetraacetic acid), which suggests its existence as an integral membrane protein (Santoni et al. 1998). Two wheat annexins accumulate in the plasma membrane in response to cold and can only be released by detergent treatment (Breton et al. 2000). The ability to exist as integral membrane proteins has given credence to the idea that plant annexins may act as transport proteins.

6 Ion Transport by Plant Annexins

Various animal annexins have been found to from Ca^{2+}-permeable conductances in vitro (Pollard and Rojas 1988; Berendes et al. 1993; Liemann et al. 1996; Kubista et al. 1999; Isas et al. 2000; Neumann et al. 2000; Golczak et al. 2001; Kirilenko et al. 2006), using planar lipid bilayers or liposomes as test systems. The sequence similarities shared by plant annexins (Fig. 1), plus the latter's membrane association, have prompted the premise that they too could form ion transport pathways. Ionic selectivity of animal annexins is conferred by salt bridges in the central core of the protein and these are sometimes conserved in plant annexins (Berendes et al. 1993; Liemann et al. 1996; Laohavisit et al. 2009; Fig. 1). In vitro transport capacity of animal annexins is variously regulated by voltage (Liemann et al. 1996; Kourie and Wood 2000), cAMP, ATP, GTP (Bandorowicz-Pikula et al. 2003; Kirilenko et al. 2006), hydrogen peroxide (Kubista et al. 1999) and low pH (Köhler et al. 1997; Langen et al. 1998; Rosengarth et al. 1998). The mechanistic basis of ion transport by animal annexins may relate to their mode of association with the bilayer and level of oligomerisation. The longstanding "electroporation" model has annexin attachment as a monomer disrupting the bilayer sufficiently to cause the passage of ions through the core of the protein (Huber et al. 1992; Hawkins et al. 2000; Kourie and Wood 2000; Golczak et al. 2001). At neutral pH, GTP can promote transport activity possibly through attachment of a trimer (Golczak et al. 2001;Kirilenko et al. 2006). In contrast, at low pH annexin insertion into the membrane is thought to underpin channel formation (Golczak et al. 2001; Hegde et al. 2006). Applying in vitro channel data to in vivo scenarios of $[Ca^{2+}]_{cyt}$ mobilisation in animal cells has proved problematic. This may be because of the annexin redundancy as transport proteins, their ability to modulate other transport proteins, the translational state of the protein or the resolution of the $[Ca^{2+}]_{cyt}$ imaging used. One of the greatest challenges facing annexin research is the detection of what could be highly transient and localised membrane interactions and transport activity.

There are few reports as yet of transport activity by plant annexins. Addition of recombinant CaANN24 to vesicles caused an increase in luminal $[Ca^{2+}]$, consistent

with the formation of a transport route (Hofmann et al. 2000a). Planar lipid bilayers have been used to demonstrate seemingly voltage-independent K^+ and possibly H^+ transport by recombinant AtANN1 at acidic pH (Gorecka et al. 2007). Its Ca^{2+}-permeation remains unknown but as a K^+ channel it could perhaps modulate membrane voltage under conditions of cytosolic acidosis resulting from stress. Work with the native maize annexin doublet ZmANN33 and ZmANN35 has shown its capacity to cause transient and dose-dependent elevation of $[Ca^{2+}]_{cyt}$ when added to root epidermal protoplasts of *Arabidopsis* expressing cytosolic (apo) aequorin as a $[Ca^{2+}]_{cyt}$ indicator (Laohavisit et al. 2009). This suggests that the maize annexin was forming a Ca^{2+} influx route directly and/or regulating native *Arabidopsis* Ca^{2+}-permeable channels (including those formed putatively by its annexins) in the plasma membrane. The pharmacological profile of the $[Ca^{2+}]_{cyt}$ transient was consistent with activation of non-selective Ca^{2+}-permeable channels (NSCC). Indeed, the maize annexin doublet was found to form a Ca^{2+}-permeable conductance in planar lipid bilayers and a similar pharmacological response and a K^+:Ca^{2+} permeability ratio typical of plant NSCC was observed (Laohavisit et al. 2009). The maize annexin current was largely independent of voltage, another feature of NSCCs. Results were obtained at acidic pH which may mean that the annexins inserted into the bilayer.

The Ca^{2+}-permeability of the maize annexin was far lower than that typically reported for animal counterparts (Laohavisit et al. 2009). Measurements from plant annexins in native membranes are now required to examine this and place activity into context. The linearity of the current–voltage relationship may reflect a dose dependency. Dose dependency has been apparent in animal annexin channel activity in lipid bilayers, affecting channel kinetics and voltage relations. High concentrations (greater than 1 nM) of animal annexin A5 supports long periods of channel opening or closing and an almost linear relationship between current and voltage (Neumann et al. 2000). Antibody addition to the opposite side of the bilayer to that exposed to the annexin abolished activity, pointing to the operation of a trans-bilayer protein (Neumann et al. 2000). At lower concentration, less than 0.1 nM, opening and closing periods shortened, activity was resistant to antibody treatment (suggesting bilayer association) and more voltage-dependent (Neumann et al. 2000). Local availability of annexin may therefore determine levels of association and channel behaviour.

7 Ligands and Interacting Partners

Plant annexins have been variously found to bind ATP, GTP and actin in vitro. The structural basis of purine nucleotide binding and hydrolysis of plant annexins (maize, tomato, cotton; McClung et al. 1994; Calvert et al. 1996; Lim et al. 1998; Shin and Brown 1999) appears to differ from animal counterparts in that it may depend upon a Walker A motif (GXXXXGKT/S) and a GTP-binding motif typical of the GTPase superfamily (DXXG) (Clark et al. 2001 and reviewed by Mortimer

et al. 2008; Fig. 1). Such sequences appear in the fourth repeat of cotton GhANN1, and GTPase activity is lost when that repeat is deleted (Shin and Brown 1999). The region for ATP/GTP binding overlaps with that for Ca^{2+}, suggesting possible competitive effects between the two ligands (Fig. 1). Site-directed mutagenesis of Ca^{2+}-binding sites had no effect on GTPase activity of soluble tomato annexin (Lim et al. 1998) but such hydrolytic activity of the wild type was inhibited by Ca^{2+}-dependent phospholipid binding. This supports the premise that precise location will affect an annexin's specific function. The in vivo importance of plant annexin phosphodiesterase activity is very poorly understood. Non-hydrolysable GTP or GDP analogues prevent stimulation of exocytosis by maize annexins in root cap protoplasts (Carroll et al. 1998). GTP can regulate Ca^{2+} channel formation by animal annexin A6 (Kirilenko et al. 2002, 2006) and it is feasible that GTP or ATP could regulate plant annexin transport function. The possible extracellular location of plant annexins has recently led to the proposal that ATP binding could promote annexin channel formation from the extracellular face of the plasma membrane (Laohavisit and Davies 2009; Shang et al. 2009). This could help account for the elevation of $[Ca^{2+}]_{cyt}$ by extracellular ATP, which is a regulator of cell viability, growth and stress responses (Clark and Roux 2009). It is also feasible that ATP hydrolysis by an extracellular annexin could contribute to regulating the former's concentration to exert concentration-dependent effects.

Tomato annexin still exhibits GTPase activity when bound to actin (Calvert et al. 1996). F-Actin binding has been proposed to be conferred by an IRI motif (in contrast to a more complex motif in the C terminus of animal annexins), but its presence does not always correlate positively with actin-binding in vitro (Calvert et al. 1996; Clark et al. 2001 and reviewed by Laohavisit and Davies 2009; Fig. 1). Plant annexin–actin association has been proposed to operate in signal transduction and exocytosis (Konopka-Postupolska 2007). *Mimosa* annexins catalyse bundling of F-actin in vitro but as Ca^{2+} was 2 mM, the physiological relevance of this result is unclear (Hoshino et al. 2004). Zucchini annexins bind F-actin and are found in association with the plasma membrane (Hoshino et al. 2004). Animal annexins are known to regulate actin dynamics directly or through actin-binding proteins (Hayes et al. 2004, 2006) and may also provide connections from the cytoskeleton to specific regions of the plasma membrane (Grewal and Enrich 2009). Whether plant annexins play similar roles and link $[Ca^{2+}]_{cyt}$ to membrane-cytoskeletal dynamics remains to be tested.

Plant annexin interactions with other proteins remains poorly explored. Animal annexins have far longer N-terminal domains than had the plant counterparts, and these form key sites for post-translational modification and protein–partner interactions (Gerke and Moss 2002). Animal annexins are also capable of interacting with C2-domain-containing proteins (Plant et al. 2000; Kheifets et al. 2006) to recruit them to specific membrane locations and/or to regulate their activity (e.g., human annexin A1 and phospholipase A_2; Kim et al. 2001). C2 is a Ca^{2+}-dependent domain capable of membrane binding, found in proteins involved in signalling, trafficking and targeting to membranes. A K/R/H-G-D motif in the annexin repeats and N terminus is thought to mediate interaction with the C2

domain. This motif is apparent in the fourth repeat of some plant annexins such as AtANN1, AtANN7 and CaANN24 and suggests possible interactions with plant C2-containing proteins such as phospholipase D in signalling. Rice annexins have been found to interact with Ste20-related protein kinase and an MAPK kinase in a pull-down assay, implicating them in Ca^{2+}-based signalling (Rohila et al. 2006). Finally, barley annexin has been found capable of interacting with 14-3-3 proteins which may be relevant to signal transduction (Schoonheim et al. 2007).

The plant annexins implicated in ion transport (AtANN1, ANNCa24, ZmANN33/35; Hofmann et al. 2000a; Gorecka et al. 2007; Laohavisit et al. 2009) plus *Brassica juncea* BjANN1 (Jami et al. 2008) have also been found capable of sustaining peroxidase activity in vitro (Gidrol et al. 1996; Gorecka et al. 2005; Laohavisit et al. 2009; Mortimer et al. 2009). The mechanistic basis for interaction with H_2O_2 remains unclear. It had been proposed that an N-terminal putative haem-binding domain centring on a conserved His40 confers activity (Clark et al. 2001; Fig. 1), but haem has yet to be detected and site-directed mutagenesis of His40 does not completely abolish activity (Gidrol et al. 1996; Gorecka et al. 2005; Konopka-Postupolska et al. 2009). It is feasible that an S3 cluster, containing two cysteine residues plus methionine and tyrosine (MCCY sulphur cluster; Hofmann et al. 2003 and Fig. 1), permits electron transfer and redox reactions. Critically, peroxidase activity can be retained on association with lipids (Mortimer et al. 2009), so, although activity in vitro is low compared with other peroxidases, annexins could be involved in Ca^{2+}-dependent redox signalling at specific membrane sites, acting to lower local concentrations of H_2O_2 (Mortimer et al. 2009; Laohavisit et al. 2009).

8 Scenarios for In Vivo Function in Plant Ca^{2+} Relations

Possible annexin functions have been reviewed recently by Mortimer et al. 2008 and Laohavisit and Davies 2009. Annexins are distributed throughout the plant during development and can be differentially distributed within a cell during its cycle (Proust et al. 2009; Clark et al. 2001; reviewed by Mortimer et al. 2008; Laohavisit and Davies 2009). Expression levels and abundance change during development and as a function of environmental stimuli such as light, pathogen attack, nutrient deprivation, salinity, drought, heavy metals and gravity (reviewed by Mortimer et al. 2008, Laohavisit and Davies 2009), clearly indicating that annexins are outputs of signalling cascades, including those regulated by $[Ca^{2+}]_{cyt}$. Annexins also appear downstream of ABA and salicylic acid (Gidrol et al. 1996; Kovács et al. 1998; Hoshino et al. 2004; Konopka-Postupolska et al. 2009). Drought tolerance is improved through overexpression of *ANNBj1* or *ANNAt1* (Jami et al. 2008; Konopka-Postupolska et al. 2009) which limits accumulation of intracellular peroxide downstream of ABA, implicating annexin peroxidase activity (Konopka-Postupolska et al. 2009). The two cysteine residues of AtANN1 which are in its S3 cluster (Fig. 1) are S-glutathionylated in response to

ABA treatment and this halves affinity for Ca^{2+} (Konopka-Postupolska et al. 2009). This could in turn affect mobilisation to membranes as a consequence of ABA-induced elevation of $[Ca^{2+}]_{cyt}$.

Annexin movement to membranes has not yet been tracked with the same level of spatio-temporal precision as for some animal annexins, but it is envisaged that an increase in $[Ca^{2+}]_{cyt}$ would cause relocation and possibly a change in spatio-temporal specific function. Peroxide, low pH and membrane hyperpolarisation are also likely to cause annexin partitioning to membranes (Hofmann et al. 1997; Gorecka et al. 2007; Laohavisit et al. 2009;Mortimer et al. 2009). *Bryonia diocia* annexin transits to the plasma membrane in response to mechano-stimulation (Thonat et al. 1997) which is likely to involve a $[Ca^{2+}]_{cyt}$ increase (Campbell and Thomson 1977). Cotton fibre annexin undergoes Ca^{2+}-dependent recruitment to the plasma membrane where it is likely to be phosphorylated by a plasma membrane-associated kinase (Andrawis et al. 1993; Shin and Brown 1999). AtANN1 undergoes substantial, Ca^{2+}-dependent relocation to root membranes after 2 h of salinity stress. This can be prevented by application of a Ca^{2+} chelator, and the bulk of this annexin is restored to the cytosol after 24 h (Lee et al. 2004). Maize annexins in roots relocate to the plasma membrane in response to humic substances (Carletti et al. 2008). From the in vitro studies described earlier, AtANN1 and the maize anexins could be acting as channels and/or peroxidases when they relocate to membranes. Maize annexins acting as channels have been postulated to catalyse the plasma membrane Ca^{2+} influx associated with cell death (Laohavisit et al. 2009). *Medicago truncatula* annexin 1 is expressed in response to nodulation factors and localises to nuclear membranes in root cells where it is proposed to contribute to local Ca^{2+} oscillations that encode downstream events in symbiotic signalling (de Carvalho-Niebel et al. 2002; Talukdar et al. 2009).

Animal annexins are implicated in Ca^{2+} flux across the plasma membrane and endomembranes (Kubista et al. 1999; Watson et al. 2004). This could be achieved by their own transport activity or through the modulation or trafficking of other Ca^{2+} transport proteins such as the ryanodine receptor (Gerke et al. 2005; Monastyrskaya and Babiychuk 2009). As Ca^{2+} transporters recruited in response to $[Ca^{2+}]_{cyt}$ elevation, plant annexins could amplify a Ca^{2+} signal, but the findings that low pH and peroxide can recruit to membranes suggests that as Ca^{2+}-permeable channels they could initiate a $[Ca^{2+}]_{cyt}$ elevation in response to such triggers. This would place them in stress-signalling cascades involving production of not only reactive oxygen species (such as salinity, Yang et al. 2007) but also in polar growth mechanisms. In polar growth, reactive oxygen species (ROS), $[Ca^{2+}]_{cyt}$ and pH are all key variables in the control of localised growth. Annexins are abundant at polar growth points such as the root hair apex (reviewed by Mortimer et al. 2008). It has been proposed that annexins may form the ROS-stimulated Ca^{2+} influx pathway at the plasma membrane that is downstream of plasma membrane NADPH oxidase-dependent ROS production in polar growth and signalling (Foreman et al. 2003; Demidchik et al. 2009; Laohavisit and Davies 2009). It is feasible that annexins associate with the plasma membrane lipid rafts that are at the apex of polar cells (Jones et al. 2006) so that their impact on $[Ca^{2+}]_{cyt}$ and ROS would be finely

spatially regulated. A *Medicago truncatula* annexin (MtANN2) has been found to co-localise with an NADPH oxidase in plasma membrane lipid rafts (Lefebvre et al. 2007), but this could reflect a structural role for the annexin. Raft-associated animal annexins connect this domain to the actin cytoskeleton, which may also be the case for plant counterparts (Konopka-Postupolska 2007).

Local modification of the bilayer by ROS could in turn influence Ca^{2+} transport characteristics of plant annexins. Malondialdehyde (found in plant membranes as a consequence of stress-induced lipid peroxidation) binds to animal annexin A5 which may help explain the involvement of A5 in peroxide-stimulated Ca^{2+} influx (Kubista et al. 1999; Balasubramanian et al. 2001). Malondialdehyde causes an inward rectification of the Ca^{2+}-current carried by maize annexins in planar lipid bilayers (Fig. 2; Laohavisit 2009). In non-oxidised bilayers the current has a near linear dependence on voltage (Laohavisit et al. 2009). ROS-induced malondialdehyde incorporation into membranes would therefore restrict annexin-mediated Ca^{2+} transport to a narrow, very negative voltage range and potentially

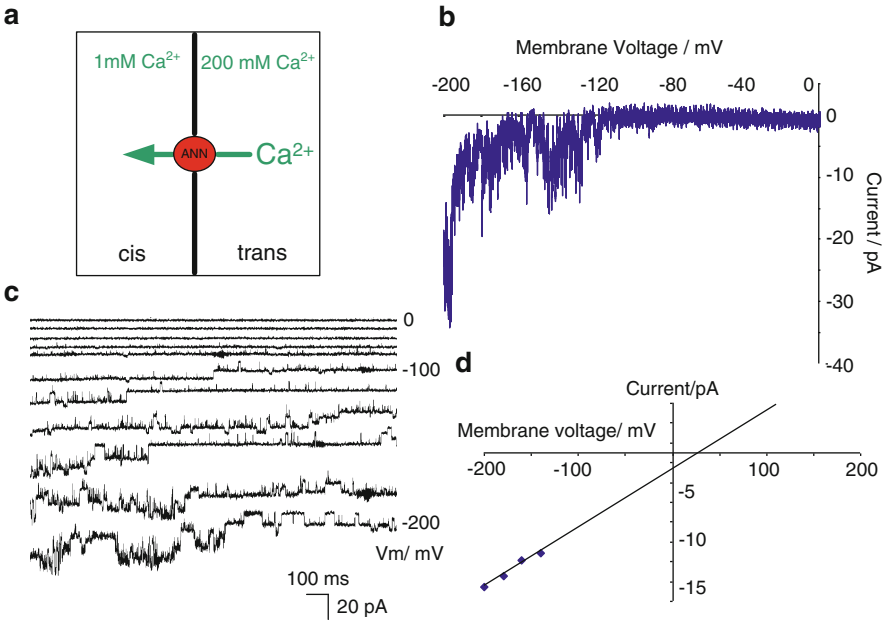

Fig. 2 Malondialdehyde promotes inward rectification of annexin-mediated Ca^{2+} current in planar lipid bilayers. (**a**) Maize annexin and lipids were prepared according to Laohavisit et al. (2009), and 3-μg protein was added to the *cis* chamber. The bilayer comprised a 5:3:2 mix of malondialdehyde-conjugated 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (Balasubramanian et al. 2001), 1-palmitoyl-2-oleoyl-phosphatidylcholine and 1-palmitoyl-2-oleoyl-phosphatidylserine. The *cis* compartment contained 1-mM $CaCl_2$, pH 4, the *trans* contained 200-mM $CaCl_2$, pH 4. (**b**) Example of inwardly rectifying current evoked by voltage ramping. Current below the voltage axis is consistent with Ca^{2+} flow from *trans* to *cis* chamber. (**c**) Example of single-channel activity observed as a function of voltage, conditions as in (**a**). (**d**) Current–voltage relationship of the first open state from (**c**). Single-channel conductance is 17 pS

modulate the $[Ca^{2+}]_{cyt}$ signal. Annexin channels could thus act at the interface between ROS and $[Ca^{2+}]_{cyt}$ in signalling and development.

9 Conclusions

With so many variables potentially regulating annexin localisation and function, elucidating their involvement in plant Ca^{2+} relations remains a formidable task. As post-translational modification of cysteines in the S3 cluster potentially places annexins downstream of ABA, H_2O_2 and nitric oxide, the generation of lines with mutations at these residues should shed light on the chain of events in which an annexin features. Real-time tracking of annexins in response to stimuli is urgently needed to identify membrane locations and dwell times during signalling and place in vitro activities into context. That annexins can interact with kinases and could interact with C2-containing proteins calls for their inclusion in interactome studies for Ca^{2+} signalling. Ca^{2+} transport capacity observed for isolated annexins now needs corroboration from studies on native membranes to secure a place for plant annexins as true modulators of plant Ca^{2+}.

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Structure and Function of CDPK: A Sensor Responder of Calcium

Yohsuke Takahashi and Takeshi Ito

Abstract Cellular Ca^{2+} signals are decoded by Ca^{2+} sensor proteins that relay the information into downstream responses. Ca^{2+}-dependent protein kinases (CDPKs) are Ser/Thr protein kinases found only in plants and some protozoans. They are sensor responders that have both Ca^{2+} sensing function and kinase activity within one protein. In plants, CDPKs comprise a large gene family and are involved in many diverse physiological processes. CDPKs are activated upon Ca^{2+} binding to their calmodulin-like domain with a large conformational change, which makes them effective switches for the transduction of Ca^{2+} signals in cells. Here we review some of the remarkable progress that has been made in recent years, especially structure and direct substrates of CDPKs.

1 Introduction

As sessile organisms, plants have acquired developmental plasticity during their evolution to integrate their endogenous program for morphogenesis with the ever-fluctuating environment throughout their life cycle. The mechanisms used to detect and institute these changes are collectively referred to as signal transduction. Ca^{2+} is a ubiquitous second messenger that is involved in the signal transduction of many environmental and developmental stimuli in eukaryotes (Sanders et al. 2002; Schuster et al. 2002). In response to diverse internal and external stimuli, cells generate transient increases in the concentration of intracellular free Ca^{2+} that vary in amplitude, frequency, duration, intracellular location, and timing (McAinsh and Hetherington 1998; Berridge et al. 2000; Allen et al. 2001; Evans et al. 2001). Different Ca^{2+} sensor proteins decode specific Ca^{2+} signatures and bring about

Y. Takahashi (✉) • T. Ito
Department of Biological Science, Graduate School of Science, Hiroshima University, 1-3-1, Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan
e-mail: ytakahas@hiroshima-u.ac.jp

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changes in metabolism and gene expression (Sanders et al. 1999, 2002; Rudd and Franklin-Tong 2001; Schuster et al. 2002).

Plant Ca^{2+} sensor proteins have been classified conceptually into sensor relays and sensor responders (Sanders et al. 2002). Sensor relay proteins, such as calmodulin (CaM), bind Ca^{2+} ions and undergo Ca^{2+}-induced conformational changes but lack out-put domains. By contrast, sensor responder proteins, for example, Ca^{2+}-dependent protein kinases (CDPKs), combine within one protein a sensing function (i.e., Ca^{2+} binding) with a response activity (i.e., protein kinase activity). The dynamic interplay between Ca^{2+} signatures and Ca^{2+} sensor proteins contributes to generating stimulus specificity of Ca^{2+} signaling.

Although many different reactions are in place to implement the proper signaling, protein phosphorylation is one of the most prominent reactions. Among protein kinases in plants, CDPKs with CBL (calcineurin B-like protein)-interacting protein kinases (CIPKs) (Kudla et al. 1999; Shi et al. 1999) are thought to play central roles in Ca^{2+} signaling because protein kinase C and conventional calmodulin-dependent protein kinase (CaMK), which represent the two major types of Ca^{2+}-regulated kinases in animal systems, are missing from *Arabidopsis thaliana* (Hrabak et al. 2003). In this review, we focus on the recent progress of studies on structure, regulation, and function of CDPK.

2 Structure

CDPKs are Ser/Thr protein kinases that are only found in plants and some protozoans (Harper et al. 1991; Suen and Choi 1991; Cheng et al. 2002; Hrabak et al. 2003). There are 34 genes encoding CDPKs in *Arabidopsis* (*Arabidopsis* Genome Initiative 2000) and 29 genes in rice (*Oryza sativa*; Asano et al. 2005). A phylogenic comparison of CDPKs suggests 12 subfamilies (Harper et al. 2004). CDPK proteins are composed of a variable N-terminal domain, a catalytic domain, a junction domain containing an autoinhibitory segment, and a calmodulin-like domain (Fig. 1). They are activated upon Ca^{2+} binding to four EF hands in the calmodulin-like domain, which makes them effective switches for the transduction of Ca^{2+} signals. Phylogenetic analyses have proposed that the CDPK gene family arose through the fusion of a CaMK and a calmodulin (Harper et al. 1991; Cheng et al. 2002). For example, the catalytic domain of tobacco NtCDPK1 is 46% identical and 75% similar to that of mouse CaMKII at the amino acid sequence level.

2.1 Variable N-Terminal Domain

Although amino acid sequences of a catalytic domain, a junction domain, and a calmodulin-like domain are highly conserved among CDPK isoforms, the variable N-terminal domain of CDPKs is diversified not only in amino acid sequence, but

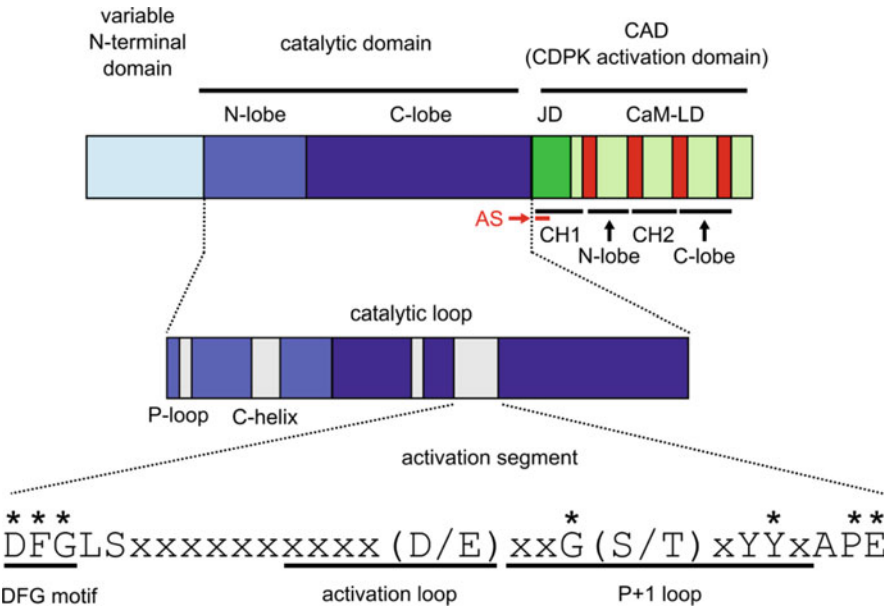

Fig. 1 Structure of CDPK. (*Top*) Schematic diagram of CDPK. CDPK protein is composed of a variable N-terminal domain, a catalytic domain, a junction domain (JD), and a calmodulin-like domain (CaM-LD). The catalytic domain (kinase domain) consists of a small N-terminal lobe of β-sheets and a larger C-terminal lobe of α-helices. The CAD consists of JD and CaM-LD. CaM-LD has four EF hands (*red stripe*). The CAD contains CH1 and CH2 helices, and N- and C-terminal EF lobes. The autoinhibitory segment (AS) is included in the CH1 helix (*red line*). (*Middle*) Schematic diagram of the catalytic domain of CDPK. The N-terminal lobe of the catalytic domain contains the phosphate-binding loop (P-loop) and C-helix. The C-terminal lobe of catalytic domain contains the catalytic loop and the activation segment. (*Bottom*) Amino acid sequence of the activation segment of CDPK. The activation segment consists of the DFG motif, the activation loop, and the P + 1 loop. Asp or Glu is located at the position analogous to Thr-197 in the activation loop of PKA, whose phosphorylation is required for full activation of PKA. Ser and Thr in the P + 1 loop are conserved in plant and parasitic CDPK, respectively. *Asterisks* represent the common amino acid residues in many Ser/Thr kinases

also in length, ranging from 25 to 180 amino acids in *Arabidopsis*. CaMK lacks an obvious variable N-terminal domain. Despite the diversity within the variable N-terminal domain, most plant CDPKs have a Gly residue at the second position. When placed in a proper context, this N-terminal Gly residue can be modified by covalent attachment of myristic acid that promotes protein–membrane interactions. Although none of the CDPKs appears to be an integral membrane protein, 24 of the 34 *Arabidopsis* CDPKs have potential *N*-myristoylation motifs for membrane association in the beginning of their highly variable N-terminal domain. The addition of a myristic acid residue is not always sufficient for membrane attachment. Often, a second lipid modification, such as palmitoylation, is necessary to stabilize the interaction with membrane. All 24 *Arabidopsis* CDPKs predicted to have a myristoylation consensus sequence also have at least one Cys residue at

position 3, 4, or 5, a potential palmitoylation site. Both modifications were actually observed in plant cells (Martin and Busconi 2000) and mutations in N-terminal Gly or Cys-affected membrane association of CDPKs. Parasitic CDPKs reserve Gly at the second position but not N-terminal Cys. Little is known about the other functions of the variable N-terminal domain of CDPKs; however, recent study with NtCDPK1 showed that the variable N-terminal domain is involved in the substrate recognition (see below, Ito et al. 2010).

2.2 Catalytic Domain

Protein kinases can be separated into two main groups: Ser/Thr-specific kinases, including CDPK, and Tyr-specific kinases. Although these kinase groups phosphorylate different residues, they have similar structures in the catalytic domain (Huse and Kuriyan 2002; Nolen et al. 2004). Classical protein kinases have a canonical catalytic domain of 250 amino acids in length, which consists of a small N-terminal lobe of β-sheets and a larger C-terminal lobe of α-helices (Ubersax and Ferrell 2007). The protein substrate binds along the cleft between the two lobes and a set of conserved residues within the catalytic domain catalyze the transfer of the phosphate of ATP to the Ser, Thr, or Tyr residue of the substrate. A slight structural difference between Ser/Thr kinases and Tyr kinases is the depth of catalytic cleft; Tyr kinases have a deeper catalytic cleft than Ser/Thr kinases. Each lobe contains conserved sequence motifs (Hanks and Hunter 1995). The N-terminal lobe contains the phosphate-binding loop (P-loop, also known as glycine-rich loop), which binds ATP, and the C-helix, whose orientation coordinates many parts of the molecule in the proper conformation necessary for optimal catalysis. The C-terminal lobe also contains conserved motifs such as the DFG motif (also known as magnesium-positioning loop) that is required for magnesium binding, the activation loop that serves as a phosphorylation-dependent activation switch in many protein kinases, the P + 1 loop that provides contacts to the site adjacent to the site of phosphorylation in the substrate, and the catalytic loop that is involved in the binding of the γ-phosphate of ATP and provides catalytic residues of the phosphoryl transfer (Fig. 1). The structure of the catalytic domain of CDPK is typical of Ser/Thr-type protein kinases (Wernimont et al. 2010).

A characteristic feature of the catalytic domain of CDPK is found in the activation loop. The activation loop of cAMP-dependent protein kinase A (PKA) must be phosphorylated at Thr-197 for full activity. When Thr-197 is phosphorylated, strong ion pairs are formed between the phosphoester and the side chains of Lys-189, also in the activation loop, and Arg-165 in the catalytic loop to form a functional active site. Interestingly, known CDPKs have either Asp or Glu at the position analogous to Thr-197 of PKA. Because both of these amino acids would be capable of mimicking phosphorylation, CDPKs exhibit full activity without phosphorylation of the activation loop.

2.3 CDPK Activation Domain

The most important advance of CDPK study in the field of structural biology in recent years is the solution of parasitic CDPK structures of the autoinhibited and activated (i.e., Ca^{2+}-bound) conformations (Wernimont et al. 2010, 2011). The structures showed that all the regions downstream of the catalytic domain (i.e., the junction domain and calmodulin-like domain) work together for activation, and so Wernimont et al. termed the entire C-terminal region the CDPK activation domain (CAD). The calmodulin-like domain is composed of two globular structural domains (N-lobe, C-lobe), each containing a pair of EF hand Ca^{2+}-binding sites. In the inactivated state, the first part of the CAD emanates from the base of the catalytic domain in the form of a long helix, named CH1, and the two lobes are separated by another long helix, named CH2 (Fig. 2). The CH1 helix contains the autoinhibitory segment, which blocks the substrate binding site of the catalytic domain and extends a basic residue, Lys-338 (with Arg being a frequent substitute for Lys), to interact with conserved acidic cluster made up of Glu-135 and Asp-138 on N-lobe of catalytic domain in parasitic CDPK (Wernimont et al. 2010). This Lys/Arg-Glu-Asp triad is conserved in parasitic and plant CDPKs and keeps important Glu in the N-terminal lobe of the catalytic domain away from the ATP binding site, in a fashion similar to the pseudosubstrate inhibition mode observed in CaMKII. Measurements of calcium-dissociation constants (*Kd*) of an *Arabidopsis* CDPK in vitro predict that *Kd* values were 0.6 μM and 30 nM for the N- and C-terminal lobes, respectively (Christodoulou et al. 2004). Because basal intracellular calcium concentrations are estimated to be ~0.1 μM, the C-terminal lobe would be completely saturated with Ca^{2+}, and thus the role for Ca^{2+} sensor in response to Ca^{2+} signatures in vivo would be assigned to the two weaker affinity binding sites in the N-terminal lobe. Comparison of the structure of autoinhibited

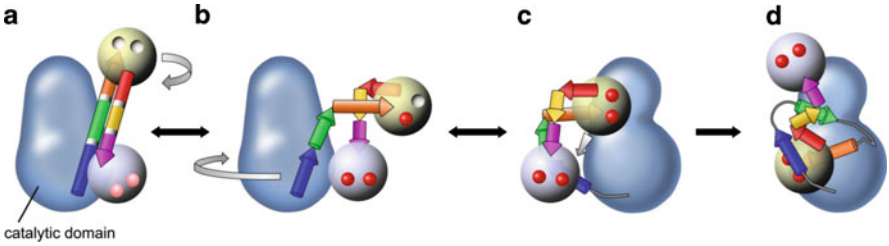

Fig. 2 Proposed mechanism of activation of CDPK. (**a**) Inactivated state of CDPK. The CH1 and CH2 helices and the N- and C-terminal EF lobes of CAD are represented as two 3D arrows and two globes, respectively. The CH1 helix contains the autoinhibitory segment, which blocks the substrate binding site of the catalytic domain (*blue region* in 3D arrow). Considering the basal intracellular Ca^{2+} concentrations, the C-terminal lobe of CAD would be saturated with Ca^{2+} in cells (*pink circle*). (**b**, **c**) Predicted conformational change in CDPK. Ca^{2+} binding allows the autoinhibitory segment to move out of the catalytic domain. *Red circles* represent Ca^{2+} ions. (**d**) Activated state of CDPK. The entire CAD is translocated to a new position roughly 135° clockwise from its inactivated position upon Ca^{2+} binding. This figure is modified from Wernimont et al. (2011)

CDPK with that of activated CDPK showed that the entire CAD is translocated to a new position roughly 135° clockwise from its inactivated position about the catalytic domain upon binding of four Ca^{2+} ions to EF hands (Wernimont et al. 2010). This movement makes the important Glu residue in the N-terminal lobe of the catalytic domain available to interact with ATP, allows the activation loop to assume the active orientation, and removes occlusion from the substrate-binding state (Fig. 2).

Ca^{2+}-binding also induces the CAD to undergo substantial refolding. The CH1 and CH2 helices are no longer antiparallel; instead, they are partially unwound and bent, and they are intricately intertwined around each other and are engaged in hydrophobic interaction with the two calcium-loaded EF lobes (Wernimont et al. 2010). Because residues involved in making the conformational change are mostly conserved between parasitic CDPKs and plant CDPKs, the overall mechanism of activation of CDPKs might be similar (Wernimont et al. 2011).

3 Regulation

A fundamental question of calcium signaling is how a simple nonprotein second messenger can regulate so many signal transduction pathways preventing unwanted crosstalk. Response specificity of individual CDPKs is believed to be generated by their varying expression pattern, their varying subcellular compartmentalization, varying calcium and lipid sensitivities, and differences in substrate recognition.

3.1 Transcriptional Regulation

Relative expression levels of *Arabidopsis* CDPKs in roots, shoots, and pollen were investigated (Harper et al. 2004). The expression of 6 out of 34 genes is of very low-level or undetected. A characteristic feature is that mRNAs for 5 CDPKs (CPK14, 16, 17, 24, 34) are highly accumulated in pollen, suggesting that multiple isoforms are expressed in a single-cell type of plants. Because effects of overexpression of these CDPKs on the growth of pollen tubes are variant (Zhou et al. 2009), each isoform might play a distinct role in pollen.

The expression of CDPK genes is also regulated by various stimuli, including, hormones (Yoon et al. 1999; Davletova et al. 2001; Kumar et al. 2004; Syam Prakash and Jayabaskaran 2006; Yu et al. 2006; Gargantini et al. 2009), NO (Lanteri et al. 2006), cold (Llop-Tous et al. 2002; Wan et al. 2007), heat (Wan et al. 2007), dark (Frattini et al. 1999), salt (Lu et al. 2006; Zhang et al. 2005; Mehlmer et al. 2010), sugar (Ohto and Nakamura 1995; Raíces et al. 2003; Martínez-Noël et al. 2007), drought (Patharkar and Cushman 2000; Liu et al. 2006; Ray et al. 2007), wounding (Szczegielniak et al. 2005; Tsai et al. 2007), and pathogen (Chico et al. 2002; Chung

et al. 2004). These observations suggest that CDPKs are involved in physiological adaptation in response to various environmental stimuli.

3.2 Ca^{2+}

Different CDPK isoforms could be activated by different amplitude of Ca^{2+}. The concentrations of Ca^{2+} required for half-maximal activity ($K_{0.5}$) for soybean CDPKα and γ were 0.06 and 1 μM, respectively (Lee et al. 1998). This could be derived from the difference of the calmodulin-like domain, in which CDPKγ has eight extra amino acids between EF hands III and IV. Furthermore, Ca^{2+} activation thresholds can vary depending on substrates (Lee et al. 1998). These mechanisms may contribute to the specificity of Ca^{2+} signaling.

3.3 Phosphorylation

Protein phosphorylation is the most widespread posttranslational modification. Many protein kinases are activated by autophosphorylation or another kinase. As stated earlier, PKA is activated by phosphorylation at Thr-197. The equivalent position in CDPKs is Asp or Glu that mimics a phospho-activated state, suggesting that CDPKs exhibit full activities without phosphorylation in the activation loop. Although phosphorylation has been observed in many CDPKs, a physiological role of phosphorylation has not been demonstrated. Autophosphorylation of CDPKs in vitro did not confer Ca^{2+}-independent activity (Lee et al. 1998; Chaudhuri et al. 1999) as observed for mammalian CaMKII (see below).

NtCDPK2 from tobacco (*Nicotiana tabacum*) participates in plant defense signaling. Immunocomplex kinase assays showed that elicitor induces an increase in enzymatic activity of NtCDPK2 with phosphorylation. Stress-inducible phosphorylation sites of NtCDPK2 were determined (Witte et al. 2010). The Ser-40 in the variable N-terminal domain of NtCDPK2 was phosphorylated by the unknown membrane-associated kinase within 2 min after stress. Although the phosphorylation of Ser-40 did not affect the enzymatic activities, it could alter the binding affinity to their targets because Ito et al. (2010) showed that the variable N-terminal domain is involved in the substrate recognition.

A complicated effect of phosphorylation on activity is observed in McCPK1 of ice plant. Autophosphorylation sites of McCPK1 were mapped to Ser-62 in the variable N-terminal domain and Ser-420 in the calmodulin-like domain between the first and second EF hands (Chehab et al. 2004). An Ala substitution at the Ser-62 or Ser-420 site resulted in a slight increase in kinase activity relative to wild-type McCPK1; however, Ala substitutions at both sites resulted in a striking decrease in kinase activity. This suggests that at least one autophosphorylation of either Ser-62 or Ser-420 may be important for the activity of the enzyme. Alternatively, the

introduced double S62A/S420A substitution may have resulted in structural alterations in the kinase itself that could account for the reduced activity of the kinase.

The in vitro autophosphorylation sites have been mapped for several CDPKs and were found with high frequency in the variable N-terminal domain (Hegeman et al. 2006). Because the conserved phosphorylation site was not found among all tested CDPKs, phosphorylation might not be essential for enzymatic activities of CDPKs. Phosphorylation of CDPK could affect intracellular localization, substrate specificity, and protein–protein interaction.

3.4 Lipid

A central player of Ca^{2+} signal transduction in animals and yeasts is protein kinase C. This kinase is activated by the combination of Ca^{2+}, diacylglycerol, and negatively charged phosphatidylserine. A few in vitro studies suggest possible regulation of plant CDPKs by lipids (Binder et al. 1994; Farmer and Choi 1999), raising an interesting possibility that CDPK could be a node of lipid-signaling and Ca^{2+} signaling. However, it remains unclear whether lipids are involved in the functional regulation of CDPKs in vivo because plant CDPKs, except for DtCPK1 from green alga Dunalliella, lack C2 domain (Ca^{2+}-dependent lipid-binding domain) that is originally found in PKC and have been identified in hundreds of eukaryotic signaling proteins.

3.5 Subcellular Localization

CDPKs are targeted to multiple cellular locations, including the cytosol, nucleus, plasma membrane, endoplasmic reticulum, peroxisomes, mitochondrial outer membrane, and oil bodies (Harper et al. 2004), which suggests diverse roles in physiological processes. Petunia (*Petunia hybrida*) CDPK1 is localized in plasma membrane of pollen tube and may play a role in regulation of pollen growth polarity. Plasma membrane localization appears to be key to the biological function of this kinase (Yoon et al. 2006). Some plant CDPKs contain a bipartite nuclear localization signal in the junction domain with a partial overlap with the autoinhibitory segment (Raichaudhuri et al. 2006). CDPK undergoes a large structural rearrangement in response to increase in Ca^{2+} concentrations. This raises the intriguing possibility that Ca^{2+} could modulate CDPK localization by revealing or occluding the region of the kinase required to mediate nuclear import. Additionally, a similar mechanism might promote the formation of a new complex with CDPK.

3.6 Comparison with CaMK

Phylogenetic analyses have proposed that the CDPK gene family arose through the fusion of a CaMK and a calmodulin (Harper et al. 1991; Cheng et al. 2002). Although both CDPK and CaMK adopt the pseudosubstrate inhibition feature, CDPKs do not share all other important mechanistic features of CaMKs. Under resting conditions CaMKII is inactive, but upon Ca^{2+}/CaM binding, a conformational change relieves the autoinhibitory effect of the regulatory domain on the catalytic domain, activating the enzyme (Hudmon and Schulman 2002; Rosenberg et al. 2005). In the sustained presence of Ca^{2+}/CaM, CaMKII undergoes intermolecular autophosphorylation at Thr-287 (or 286; specific numbering is isoform dependent), resulting in Ca^{2+}/CaM-independent activity (Hudmon and Schulman 2002). Thr-287 lies within the autoinhibitory segment of CaMKII, and autophosphorylation at Thr-287 produces Ca^{2+}-autonomous activity by preventing reassociation of the catalytic domain by the autoinhibitory segment (Hudmon and Schulman 2002). Although the autoinhibitory segment of CDPKs shows similarity to that of CaMKII, Thr/Ser is missing at the corresponding position of CDPKs, suggesting the lack of Ca^{2+}-independent active state by autophosphorylation in the autoinhibitory segment of CDPK.

Reactive oxygen species (ROS) play important roles in regulating cell activities. CaMKII is also activated by ROS. The oxidation of paired methionine residues (Met-281/Met-282 in CaMKIIδ) in the autoinhibitory segment sustains CaMKII activity in the absence of Ca^{2+}/CaM by a mechanism analogous to autophosphorylation at Thr-287 (Erickson et al. 2008). Although the paired Met or Cys motif is conserved in CaMKII isoforms (α to δ), CDPKs do not contain the motif at the corresponding position.

A fundamental structural difference between CaMKII and CDPK is that CaMKII assembles into a dodecameric holoenzyme by the association domain of C-terminus, whereas there is no evidence for CDPK to form an oligomeric complex. CaMKII is able to respond to Ca^{2+} signals in a manner that depends on the frequency with which Ca^{2+} levels rise and fall (De Koninck and Schulman 1998). The sensitivity of CaMKII to the frequency of Ca^{2+} pulses might depend on the dodecameric structure of CaMKII holoenzyme (Rosenberg et al. 2005). Another structural difference is that movement of the autoinhibitory segment in response to Ca^{2+} signals. As stated earlier, the α-helix containing the autoinhibitory segment in CDPK is bent on Ca^{2+} binding; however, the α-helix of regulatory domain in CaMKII is maintained on the release of the inhibition. Although CDPK and CaMKII have a common ancestor, molecular mechanisms for the functional regulation might diversify during evolution to adapt to a variety of environmental stimuli characteristic to plants and animals, respectively. However, unexpected common feature has been observed. Mammalian CaMKII acts as a scaffold to recruit proteasomes to dendritic spines in addition to kinase activities (Bingol et al. 2010). Because the interaction between a regulatory subunit of the 26S proteasome and CDPK was reported (Lee et al. 2003), CDPK could play a similar role in localization of proteasome.

4 Biological Functions and Substrates

4.1 Functions

CDPKs have been reported to be involved in diverse physiological processes, including the accumulation of storage starch and protein in immature seeds of rice (Asano et al. 2002), tolerance to cold, salt, and drought stress in rice (Saijo et al. 2000), a defense response in tobacco (Romeis et al. 2001), root development and regulation of nodule number in *Medicago truncatula* (Ivashuta et al. 2005), abscisic acid (ABA) response in *Arabidopsis* (Choi et al. 2005), stomata closure in *Arabidopsis* (Mori et al. 2006), pollen tube growth in Petunia (Yoon et al. 2006), regulation of a transcription factor in tobacco (Ishida et al. 2008), regulation of ROS production in potato (Kobayashi et al. 2007), and invasion to host cells in parasite (*Toxoplasma gondii*; Kieschnick et al. 2001). To demonstrate that a CDPK plays a role in a biological process, analysis of loss-of-function mutants or knock-down study is indispensable. Recently, forward and reverse genetic approach allowed the correct assignment of a physiological function to each CDPK.

Transient loss-of-function studies by gene silencing of the NtCDPK2 subfamily showed reduction of hypersensitive response after race-specific Avr9 elicitation (Romeis et al. 2001). In complementary gain-of-function experiments, the ectopic expression of a truncated NtCDPK2 variant lacking the entire C-terminal CAD induced enhanced stress responses (Ludwig et al. 2005). Antisense expression of a rice CDPK, called SPK, resulted in defective accumulation of starch. The in vitro kinase assay showed that sucrose synthase might be a substrate of SPK (Asano et al. 2002). It has been reported that six *Arabidopsis* CDPKs, CPK3, CPK4, CPK6, CPK10, CPK11, and CPK23 are involved in ABA signaling. In single and double mutants of *Arabidopsis cpk3* and *cpk6*, ABA and Ca^{2+} activation of slow-type anion channels and ABA activation of plasma membrane Ca^{2+}-permeable channels were impaired in guard cells (Mori et al. 2006). The *Arabidopsis* mutant *cpk23* showed enhanced tolerance to drought and salt stresses, potentially due to a decrease in stomatal apertures, while the *AtCPK23* overexpression lines became more sensitive to drought and salt stresses (Ma and Wu 2007). By contrast, the *Arabidopsis* mutant *cpk10* showed a higher sensitivity to drought stress, while the *CPK10* overexpression lines displayed enhanced tolerance to drought stress. Induction of stomatal closure and inhibition of stomatal opening by ABA and Ca^{2+} were impaired in the *cpk10* mutants (Zou et al. 2010). Loss-of-function mutations of *Arabidopsis* CDPKs *CPK4* and *CPK11* resulted in pleiotropic ABA – insensitive phenotypes in *Arabidopsis* (Zhu et al. 2007). The in vitro kinase assay suggests that ABA-responsive transcription factors, ABF1 and ABF4, are possible substrates of CPK4 and CPK11. *Arabidopsis* CDPKs CPK17 and CPK34 are highly expressed in pollen. Loss-of-function mutations of *CPK17* and *CPK34* cause a severe disruption in pollen tube tip growth and tropism, resulting in nearly complete male sterility (Myers et al. 2009). RSG (for REPRESSION OF SHOOT GROWTH) is a tobacco transcriptional activator that is involved in the feedback regulation of gibberellin

(GA) (Fukazawa et al. 2000, 2010). Suppression of a tobacco CDPK, NtCDPK1, by RNA interference inhibited the GA-induced phosphorylation of Ser-114 of RSG in plants. Overexpression of NtCDPK1 inhibited the feedback regulation of a GA 20-oxidase gene (Ishida et al. 2008). GA-induced phosphorylation by CDPK was also reported in rice (Khan et al. 2005). Together, these findings point to a critical role of CDPK-mediated Ca^{2+} signaling in diverse physiological processes. The identification of direct target proteins of CDPK allows us to address more precisely how specific CDPKs contribute to a specific decoding of distinct Ca^{2+} signatures.

4.2 Substrate

The consensus phosphorylation site of CDPK is proposed to be basic-hydrophobic (Φ)-X-basic-X-X-Ser/Thr-X-X-X-Φ-basic (Harper and Harmon 2005). However, the presence of a consensus phosphorylation site in a protein does not guarantee that the protein is a substrate in vivo, and authentic phosphorylation sites do not always conform to the consensus (Ubersax and Ferrell 2007). For example, yeast cyclin-dependent kinase-1 (Cdk1) or yeast casein kinase 2 (YCK2) phosphorylated hundreds of proteins among thousands of yeast total proteins in vitro (Ubersax et al. 2003; Ptacek et al. 2005). Thus, in addition to in vitro kinase assay, more detailed experiments are required to identify physiological substrates of protein kinases. Such experiments include the investigation of the effects of knock-down and overexpression of a kinase on the phosphorylation status of target amino acid residues and of in vivo interaction between kinase and substrate. However, only a few proteins are demonstrated as physiological substrates of CDPKs at present.

NADPH oxidase plays a central role in the oxidative burst in plant disease resistance. Potato CDPK, StCDPK5, phosphorylated Ser-82 and Ser-97 in the NADPH oxidase in vitro. Ectopic expression of the constitutively active form of StCDPK5 provoked ROS production in *Nicotiana benthamiana* leaves. The CDPK-mediated ROS production was disrupted by knockdown of NADPH oxidase in *N. benthamiana*. This suppression was complemented by heterologous expression of wild-type potato NADPH oxidase but not by a mutant (S82A/S97A). Furthermore, the heterologous expression of StCDPK5 phosphorylated Ser-82 of potato NADPH oxidase in *N. benthamiana* (Kobayashi et al. 2007). These results suggest that NADPH oxidase is a physiological substrate of StCDPK5.

A tobacco transcription factor RSG is negatively regulated by 14-3-3 signaling proteins (Igarashi et al. 2001). The 14-3-3 proteins bind to RSG depending on the RSG phosphorylation of Ser-114 and thereby sequester RSG in the cytoplasm so that it is unable to regulate its target genes in the nucleus (Ishida et al. 2004). NtCDPK1 was identified as an RSG kinase that promotes 14-3-3 binding to RSG by phosphorylation of Ser-114 of RSG. NtCDPK1 interacts with RSG in vivo and in vitro and specifically phosphorylates Ser-114 of RSG in vitro. Knockdown of NtCDPK1 by RNAi repressed the GA-induced phosphorylation of Ser-114 of RSG, while overexpression of NtCDPK1 in transgenic plants promoted

phosphorylation of Ser-114 (Ishida et al. 2008). These results showed that RSG is a direct target of NtCDPK1.

4.3 Substrate Specificity

The first level of substrate specificity arises from the interaction between the active site of the kinase and the amino acid sequences surrounding the phosphorylation site of the substrate. Additional conserved docking motifs on the substrate that interacts with specific regions of the catalytic domain may increase the selectivity of the kinase substrate (Sharrocks et al. 2000). However, because the sequences of phosphorylation sites and docking motifs are rather simple and ambiguous, they are insufficient to account for the substrate specificity. Other molecular mechanisms are required to select the functional targets among potential phosphorylation sites. Scaffold proteins, such as Ste5 for yeast MAPK cascade, or targeting subunits, such as cyclin for CDK (Schulman et al. 1998), which help to enhance substrate specificity, were not found for CDPKs. The primary structures of CDPK isoforms are highly conserved, especially within their catalytic domains. Thus, it was considered unlikely that CDPKs would have distinguishable substrate specificities. However, several studies using loss-of-function mutants and knockdown plants suggested a distinct physiological function for each CDPK isoform in plants. There should be a mechanism by which the substrate is specifically recognized by a CDPK.

Ito et al. (2010) found that the variable N-terminal domain of NtCDPK1 plays an essential role in the specific recognition of substrate RSG. The recognition by the variable N-terminal domain of NtCDPK1 may strictly determine the substrate specificity, in concert with the interaction between the catalytic domain of NtCDPK1 and the phosphorylation site of the substrate. Alternatively, the interaction between RSG and the variable N-terminal domain of NtCDPK1 could result in an exposure of target site Ser-114 of RSG and help to orientate it correctly in the active site of NtCDPK1 through a conformational change in RSG. The variable N-terminal domain of NtCDPK1 conferred sufficient RSG kinase activities to an *Arabidopsis* CDPK, AtCPK9, that only poorly phosphorylates RSG (Ito et al. 2010). This results open the possibility of engineering the substrate specificity of CDPK by manipulation of the variable N-terminal domain, which would provide an approach for the rewiring the signaling pathway.

5 Future Directions

The tremendous progresses have been made in our understanding of CDPKs during the past 10 years. Nevertheless, when comparing research of CDPK with that of protein kinases in animals, the development of specific inhibitors for CDPK is

retarded. Calmodulin antagonists, including W-7, compound 48/80, and trifluoperazine, have been used to inhibit activities of CDPK. However, these compounds cannot rule out the possibility of participation of other calcium-regulated kinases, including CBL-regulated CIPK (Kudla et al. 2010), and CRK (for CDPK-related kinase) (Zhang and Lu 2003). Apicomplexan parasites are a diverse group of protozoan parasites, several of which cause important human and animal diseases, including malaria. Because humans do not have CDPKs, CDPK-specific inhibitors would be effective drugs against apicomplexan parasites. *Toxoplasma gondii* CDPK (TgCDPK1), which plays a key role in parasite invasion, contains a unique sequence variation in the ATP-binding pocket of the catalytic domain: namely, a Gly at the so-called "gatekeeper position." This variation distinguishes TgCDPK1 from other kinases, including TgCDPK3, and plant CDPKs. BKIs (for bumped kinase inhibitors) are analogs of 4-amino-1-*tert*-butyl-3-phenylpyrazolo[3,4-D] pyrimidine) that are derivatized at the C3 position with bulky aromatic groups (Bishop et al. 1999). Large gatekeeper residues, such as Met, severely restrict the access of BKIs to the ATP-binding pocket, whereas small gatekeeper residues, such as Gly present in TgCDPK1, allow the access of BKIs to the ATP-binding pocket. BKIs inhibited both activities of TgCDPK1 and parasite invasion to human cells without affecting kinases of host cells (Ojo et al. 2010). Plant CDPKs are insensitive to BKIs; however, replacement of a plant CDPK gene with its mutant version in which the large amino acid residue at the gatekeeper position is substituted to Gly, would allow phenotypic investigation of the effects of the specific inhibition of the CDPK by BKIs. This is the so-called ASKA (for analog-sensitive kinase allele) approach (Bishop et al. 2000). Alternatively, because the junction domain and calmodulin-like domain of CDPK work together on Ca^{2+} binding unlike other proteins containing EF hands, including CaM (Wernimont et al. 2010), this unique mechanism of activation could be the effective target to inhibit CDPKs selectively. Inducible knockdown of a CDPK by RNAi might be useful to reveal functions of CDPKs in vivo, since the complete removal of CDPKs that play critical roles in development might result in lethality.

Although CDPKs have been reported to be involved in diverse physiological processes, very limited information is available about the direct substrates in vivo. Ito et al. (2010) showed that the variable N-terminal domain of NtCDPK1 is directly involved in the recognition of the target protein. In *Arabidopsis*, CDPKs comprise a protein family with 34 members, all of which, except for CPK26, have a variable N-terminal domain consisting of 25–180 amino acids in length. Yeast two-hybrid analysis suggested that the variable N-terminal domain of an *Arabidopsis* CDPK, CPK32, participates in the interaction with transcription factor ABF4 (Choi et al. 2005). If the variable N-terminal domain of other CDPKs, as well as that of NtCDPK1, play roles in substrate recognition, the search for interacting proteins of the variable N-terminal domain by yeast two-hybrid screen or the TAP tag purification method would provide important clues to identify the physiological substrates of each CDPK. Phosphoproteomics has enabled large-scale identification of protein phosphorylation sites, benefiting from advances in phosphor peptide enrichment and improvements in mass spectrometry (Villén and Gygi 2008).

The comparison of phosphorylation sites after induction and repression of a CDPK in cells would be highly informative. Such comprehensive studies would improve our understanding of both the physiological roles of each CDPK and the complicated network of Ca^{2+} signaling in plants.

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Elucidation of Calcium-Signaling Components and Networks

Irene S. Day and A.S.N. Reddy

Abstract Calcium signaling depends on proteins at three nodes: generation of Ca^{2+} signature, sensing of changes in cellular Ca^{2+} level, and transduction of a Ca^{2+} signal. Plant cells have multiple mechanisms for generating increases in $[Ca^{2+}]_{cyt}$ suggesting the capacity to produce complex spatiotemporal patterns of $[Ca^{2+}]_{cyt}$ elevation. A large number of Ca^{2+} sensors have been identified experimentally or have been predicted on the basis of sequence similarity to known Ca^{2+}-binding proteins or the presence of Ca^{2+}-binding domains. The number of target proteins is expected to be large, as a given sensor can interact with multiple proteins. Proteins involved in Ca^{2+}signaling in plants have been identified using Ca^{2+}-binding, protein–protein interaction, yeast two-hybrid, and coprecipitation screens. With the completion of genomic sequencing of several plants, researchers have identified many Ca^{2+} sensors and target proteins on a global scale. In Arabidopsis, about 3–4% of the proteome appears to participate in Ca^{2+} signaling. The challenge now is the elucidation of the function of each verified/predicted protein involved in Ca^{2+} signaling on a local and global scale.

1 Introduction

Calcium is an important second messenger for relaying signals in plant-signaling pathways. The Ca^{2+} concentration in the cytoplasm ($[Ca^{2+}]_{cyt}$) is maintained at a low concentration (100–200 nM) so as not to be toxic to the plant cell, while in the cell wall and vacuole, the concentration can be millimolar (Hirschi 2004; Reddy and Reddy 2000; Rudd and Franklin-Tong 2001). A change in $[Ca^{2+}]_{cyt}$ (levels can be elevated up to 3 μM) is implicated in the regulation of many abiotic and biotic

I.S. Day • A.S.N. Reddy (✉)
Department of Biology, Program in Molecular Plant Biology, Program in Cell and Molecular Biology, Colorado State University, Fort Collins, CO 80523, USA
e-mail: iday@colostate.edu; reddy@colostate.edu

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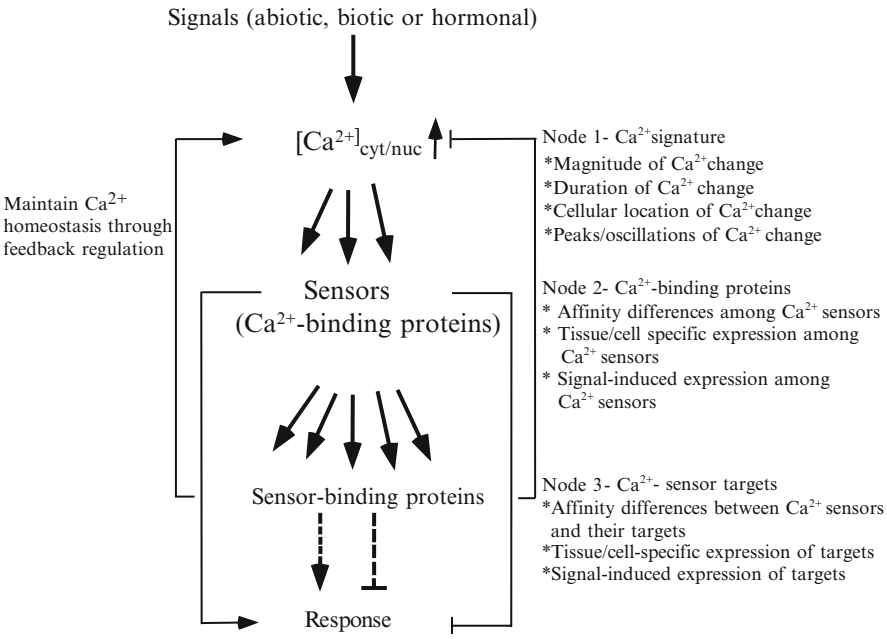

Fig. 1 Major nodes involved in cell signaling via Ca^{2+}. The three nodes each involve an increasing number of proteins. A signal causes the $[Ca^{2+}]_{cyt}$ to increase, Ca^{2+}-binding proteins (sensors) present in the cell bind Ca^{2+} according to their affinity profile and either cause a response themselves or bind to other proteins (targets) present in the cell dependent on their affinity. Various factors that contribute to specificity of signal-induced response at each node are indicated with asterisks. Also shown is the feedback regulation of Ca homeostasis by Ca^{2+} sensors and their targets

stress responses as well as several developmental processes (Reddy 2001; White and Broadley 2003). The change in $[Ca^{2+}]_{cyt}$ can be elicited by numerous abiotic and biotic stresses as well as developmental and hormonal cues. The cell-signaling process via Ca^{2+} has three major nodes/hubs: generation of a Ca^{2+} signal, sensing the signal, and transduction of the signal (Fig. 1) (Reddy and Reddy 2004a).

1.1 Generation of Ca^{2+} Signal

$[Ca^{2+}]_{cyt}$ levels are highly dynamic and regulated in response to various signals by channels, pumps, and transporters (Miedema et al. 2001). Signals lead to an increase in $[Ca^{2+}]_{cyt}$ in the form of transient spikes and oscillations (McAinsh and Pittman 2009). The magnitude, type, duration, and cellular location of the Ca^{2+} change, referred to as the Ca^{2+} signature, vary depending on the signal or cell type. Various types of Ca^{2+}-permeable channels on the plasma membrane and organellar membranes elevate $[Ca^{2+}]_{cyt}$ levels in response to diverse signals. These channels

allow Ca^{2+} to flow either from the cell exterior or from the internal stores to the cytoplasm and then the Ca^{2+} pumps and H^+/ Ca^{2+} antiporters restore the resting $[Ca^{2+}]_{cyt}$. Several types of Ca^{2+}-permeable channels elevate the $[Ca^{2+}]_{cyt}$ level in response to signals including voltage and ligand-gated channels on vacuolar, plasma, and ER membranes (Reddy and Reddy 2004a). Studies have shown that cyclic nucleotide gated channels (CNGCs), glutamate receptor family members (GLRs), and a tonoplast-localized nonselective cation channel (TPC1) are involved (Ma et al. 2009). Evidence of CNGCs involvement in Ca^{2+} homeostasis include yeast complementation of a Ca^{2+} uptake deficient mutant by CNGC1, 11, and 12 (Urquhart et al. 2007) as well as the expression of CNGC18 in *E. coli* resulting in increased Ca^{2+} accumulation (Ali et al. 2006; Frietsch et al. 2007). Work by Qi et al. (2006) indicates that GLR3.3 mediates Ca^{2+} entry into the cytosol and the *glr3.3* mutant shows impaired glutamate-mediated Ca^{2+} rise in the cytosol. It has been reported that TPC1's major function is to conduct Ca^{2+} current across the tonoplast out of the vacuole, resulting in cytosolic Ca^{2+} rise (Peiter et al. 2005); however, it does not play an important role in mediating the Ca^{2+} signal induced by different types of stresses (Ranf et al. 2008). In order for small influxes through channels to bring about significant changes in $[Ca^{2+}]_{cyt}$, the $[Ca^{2+}]_{cyt}$ needs to be maintained at sufficiently low levels. Restoration of the signal-induced $[Ca^{2+}]_{cyt}$ to the resting level and replenishment of intracellular and extracellular Ca^{2+} stores to maintain low resting $[Ca^{2+}]_{cyt}$ are accomplished by adenosine triphosphate (ATP)-fueled pumps (Ca^{2+}-ATPases) and transporters driven by the electrochemical gradients of other ions, particularly H^+ in higher plants (H^+/Ca^{2+} exchangers) (Tuteja and Mahajan 2007; Hetherington and Brownlee 2004). These pumps and transporters may also be important in determining the peak amplitudes and duration of Ca^{2+} transients. Recent reviews on proteins involved in shaping the Ca^{2+} signal (McAinsh and Pittman 2009; Tuteja and Mahajan 2007; Hetherington and Brownlee 2004) and chapter "La Calcium, C'est La Vie" by Anthony J. Trewavas of this book provide more details concerning the generation of the Ca^{2+} signal.

1.2 Sensing the Ca^{2+}

The change in $[Ca^{2+}]_{cyt}$ is sensed by Ca^{2+}-binding proteins, the Ca^{2+} sensors. Figure 2a shows schematic diagrams of representative Ca^{2+} sensors. The properties that are essential for Ca^{2+}-binding proteins include binding sites that are unoccupied at resting $[Ca^{2+}]_{cyt}$ levels and occupied at levels reached upon stimulation; selectivity and preference for Ca^{2+} over other cations; ability to undergo conformational change upon binding Ca^{2+}; very fast kinetics to correspond with the short lived Ca^{2+} signature; and interaction of Ca^{2+}-bound sensors with other proteins in most cases (Tuteja and Mahajan 2007; Krebs and Heizmann 2007). The sensors themselves may become active and transduce the signal by their activity or may bind to other proteins affecting their activity and, thereby, transduce the signal through their interacting proteins. In general, Ca^{2+} binds to a conserved motif in the Ca^{2+} sensors,

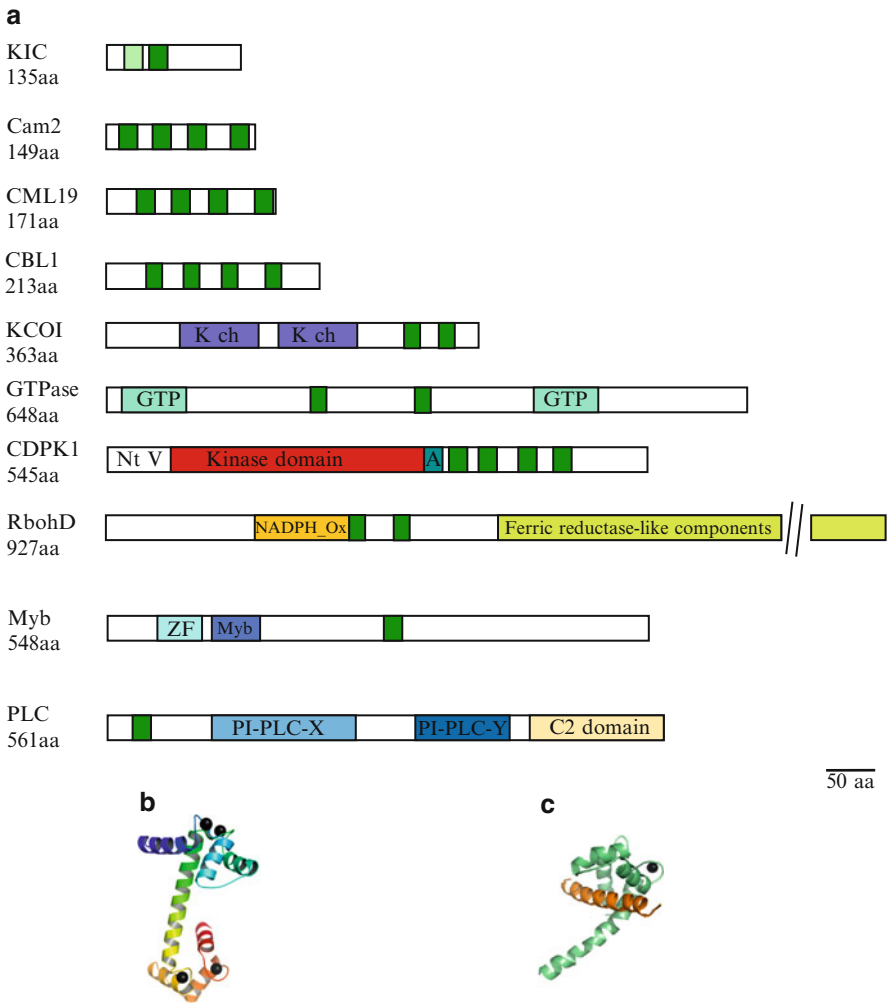

Fig. 2 Ca^{2+} sensors. (**a**) Schematic diagrams of representative Ca^{2+} sensors. *Green boxes* represent the EF hand domains, *light green box* is the non-Ca^{2+}-binding EF-like hand in KIC, other *boxes* are as labeled. K Ch, potassium channel; GTP, GTPase domain; Nt V, N-terminal variable domain; A, autoinhibitory domain; ZF, zinc finger; Myb, Myb DNA binding; PI-PLC, phospholipase C, phosphatidylinositol-specific, X or Y region. KIC, At2g46600; CaM2, At2g41110; CML19, At4g 37010; CBL1, At4g17615; KCOI, At5g55630; GTPase, At3g05310; CDPK1, At1g18890; RbohD, At5g47910; Myb, At3g07740; PLC, At5g58670. (**b**) Calmodulin showing the four bound Ca^{2+} ions (*black spheres*). (http://commons.wikimedia.org/wiki/File:Calmodulin-Ca.png) (**c**) KIC structure showing the one Ca^{2+} ion bound to the canonical EF hand. The *orange ribbon* is the KCBP CaM-binding domain that interacts with KIC (Vinogradova et al. 2009)

the most common of which is an EF-hand motif that will be discussed in detail in Sect. 2.1. A second motif is the annexin fold in members of the annexin subfamily which have been shown to be involved in stress response (Clark and Roux 1995; Konopka-Postupolska et al. 2009). Annexins are discussed in the chapter titled Annexins. Another family of Ca^{2+} sensors has a C2 domain containing 130–145 conserved amino acids, the members of which are membrane-associated proteins (Sect. 2.2). Other Ca^{2+} sensors with no conserved EF hand, annexin, or C2 domains have been identified as Ca^{2+} binding in the literature and will be discussed in Sect. 2.2.

1.3 Transduction of the Signal

Some Ca^{2+} sensors such as the CDPKs discussed in the chapter on CDPKs have domains that are regulated when they bind Ca^{2+} and the signal is responded to by these proteins. Others do not have any other recognizable domain and act by interaction with other proteins. When these Ca^{2+} sensors bind Ca^{2+} their confirmation is altered allowing interaction with other proteins, Ca^{2+} sensor target proteins, activating or inactivating them. The action of the sensor target results in response to the Ca^{2+} signal. In some cases, families of Ca^{2+} sensors interact with families of target proteins (Popescu et al. 2007). One major family of Ca^{2+} sensors, the calmodulin family and its known interacting targets, is discussed in chapter "Decoding of Calcium Signal Through Calmodulin: Calmodulin-Binding Proteins in Plants" of this volume and a second family of sensors, the CBLs and their targets, the CIPKs, is discussed in chapter "The CBL–CIPK Network for Decoding Calcium Signals in Plants". Other target proteins will be discussed further in this chapter.

1.4 Proteomics of Ca^{2+}-Signaling Components

Calcium signaling depends on proteins at all three nodes. The number of proteins involved at each node is numerous. Plant cells have multiple mechanisms for generating increases in $[Ca^{2+}]_{cyt}$ suggesting the capacity to produce highly complex spatiotemporal patterns of $[Ca^{2+}]_{cyt/nuc}$ elevation (Hetherington and Brownlee 2004). Downstream of the stimulus-induced $[Ca^{2+}]_{cyt/nuc}$ increase, the cell possesses an array of proteins that can respond to these changes. A large number of Ca^{2+} sensors have been either identified experimentally or have been predicted on the basis of homology to known Ca^{2+}-binding proteins or the presence of domains known to bind Ca^{2+} (Day et al. 2002; Boonburapong and Buaboocha 2007; McCormack et al. 2005). The number of target proteins is expected to be large, as a given sensor can interact with multiple proteins as will be discussed in Sect. 3. Further complexity arises with the involvement of other intracellular

messengers and signaling proteins that can modulate Ca^{2+} signaling (Hetherington and Brownlee 2004). Proteins involved in Ca^{2+} signaling in plants have been identified and characterized using Ca^{2+}-binding screens, protein–protein interaction screens, yeast two-hybrid assays, and coprecipitation of interacting proteins. With the completion of genomic sequencing of several plants, researchers have identified families of known Ca^{2+} sensors and target proteins on a global scale. The number of proteins involved increase from node 1 to node 3. In Arabidopsis at least 3–4% of the proteome appears to participate in Ca^{2+} signaling. The challenge now is the elucidation of the function of each verified/predicted protein involved in Ca^{2+} signaling on a local and global scale.

2 Ca^{2+} Sensors

2.1 EF-Hand Ca^{2+} Sensors

A majority of Ca^{2+} sensors have one or more EF-hand motif(s) that are responsible for binding Ca^{2+}. The EF-hand motif is a helix–loop–helix first characterized in parvalbumin (Kretsinger and Nockolds 1973). The EF nomenclature referred to helices E and F of six helices in three pairs of helix–loop–helix motifs in parvalbumin. Most known EF-hand-containing proteins have pairs of EF-hand domains, having 2–12 EF hands. The pairs form a discrete domain and many display positive cooperativity (Gifford et al. 2007). However, some EF-hand proteins have odd numbers (1, 3, or 5) (Krebs and Heizmann 2007; Day et al. 2002). The Ca^{2+}-binding loop commonly contains 12 residues usually starting with an aspartate and ending with a glutamate although three of them (10–12) including the glutamate are technically in the helix following the loop (Grabarek 2006; Gifford et al. 2007; Krebs and Heizmann 2007). The Ca^{2+} ion is coordinated in the EF-hand loop in a pentagonal bipyramidal configuration and is chelated by seven oxygen atoms contributed by residues 1, 3, 5, 7, 9, and 12 (2 atoms) (Krebs and Heizmann 2007). The residues providing ligands for Ca^{2+} coordination are referred to as + X (1), +Y (3), +Z (5), −Y (7), −X (9), and –Z (12). Residue 6 is an invariant glycine because of a sharp bend necessitated by Ca^{2+} binding to a side-chain oxygen of residue 5 and a backbone carbonyl of residue 7. Figure 3a shows an alignment of a few EF hands from Arabidopsis Ca^{2+} sensors. The differences in the amino acids in the EF-hand domains are significant enough to give each EF hand distinct biochemical properties (Bhattacharya et al. 2004). Different EF hands have different Ca^{2+} affinities as can be seen in CaM whose four EF hands are in two pairs (Fig. 3a). The C-terminal pair (EF3 and 4) binds Ca^{2+} with a K_d of approximately 10^{-6}, while the N-terminal pair (EF1 and 2) binds Ca^{2+} with a K_d of approximately 10^{-5} – a tenfold difference (Forsen et al. 1991). Proteins with EF hands are in an apoprotein form in quiescent cells; when $[Ca^{2+}]_{cyt}$ increases they bind Ca^{2+} and their conformation changes. Some EF hands can also bind Mg^{2+} (for example, the

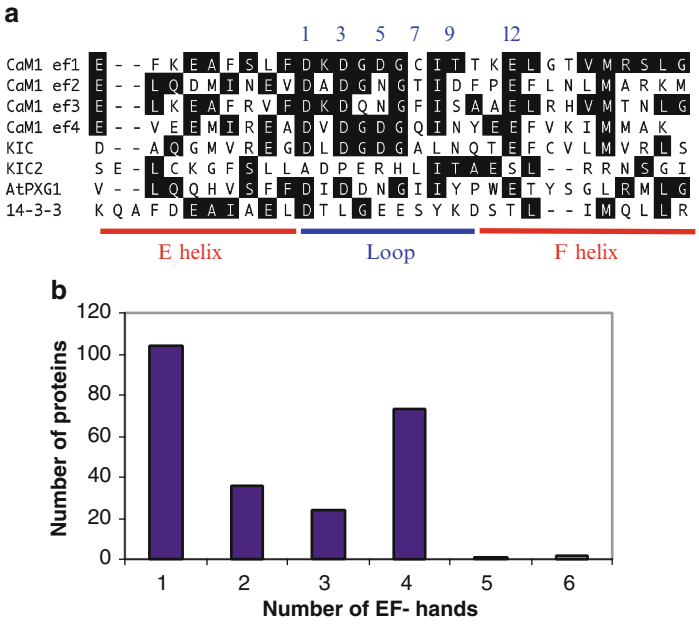

Fig. 3 EF-hand sequence alignment and numbers. (**a**) EF-hand (or EF-hand-like) domains from CaM1, KIC, and AtPXG1 and a 14-3-3 protein are aligned and the amino acids in the loop involved in Ca^{2+} binding are identified above the alignment. (**b**) The number of proteins in the Arabidopsis genome having 1–6 EF-hand motifs (Reddy and Day 2001)

third and fourth EF hands of troponin C bind Ca^{2+}/Mg^{2+}), whereas the first and second EF hands are Ca^{2+} specific (Allouche et al. 1999; Gifford et al. 2007). Ca^{2+}/Mg^{2+} discrimination relies on the affinities of the EF hands for these cations, which is dependent on the types of amino acid residues in the binding loop (Allouche et al. 1999; Gifford et al. 2007).

2.1.1 EF-Hand Proteins in Arabidopsis and Rice

Following the completion of the Arabidopsis genome sequence, we mined the genome for genes encoding EF-hand-containing proteins and identified approximately 250 proteins containing one or more EF-hand motif (Day et al. 2002) (Table 1). As stated above, most EF-hand proteins have pairs of EF hands, which facilitate the binding of Ca^{2+}. We found a large number of proteins with an odd number of EF-hand motifs (1, 3, or 5, see Fig. 3b). The proteins with an odd number of EF-hand domains may function as homo- or heterodimers, may bind Ca^{2+} in a weaker manner, there may be an unidentifiable, but functional, "cryptic" Ca^{2+}-binding motif, or may not bind Ca^{2+} at all. KCO1 (At5g55630) was identified as a Ca^{2+}-binding phosphate channel with two EF hands (Czempinski et al. 1997). However, analysis of the sequence for KCO1 by InterProScan shows one EF

Table 1 Ca^{2+} sensors

Type of sensor	# in Arabidopsis	References
CaMs	7	McCormack et al. (2005), Day et al. (2002)
CMLs	50	McCormack et al. (2005), Day et al. (2002)
CDPKs	34	Cheng et al. (2002)
RbOHs	10	Keller et al. (1998), Torres and Dangl (2005)
CBLs	10	Weinl and Kudla (2009)
Potassium channels	3	Czempinski et al. (1997), Day et al. (2002)
GTPases	3	Jayasekaran et al. (2006b), Day et al. (2002)
Centrins	2	Cordeiro et al. (1998), Molinier et al. (2004)
NADPH dehydrogenase	3	Giesler et al. (2007)
Calreticulin/calnexin	5	Jia et al. (2009), TAIR
Caleosins	8	Naested et al. (2000), TAIR
SUBS	3	Guo et al. (2002)
14-3-3	8	Athwal and Huber (2002), Day et al. (2002)
Others	104	Day et al. (2002)
Total	250	

hand, the second EF hand tandem to the first as identified by Czempinski et al. (1997) is noncanonical but may function to bind Ca^{2+} in concert with the canonical EF hand. Fimbrin (At4g26700) was originally identified as an actin cross-linking protein with one EF-hand motif (McCurdy and Kim 1998). Later research showed that it is Ca^{2+} independent (Kovar et al. 2000). A protein (ABI1, At4g26080) with a high similarity to protein serine or threonine phosphatases of type 2C also has a putative single EF-hand but was later found not to be responsive to Ca^{2+} (Meyer et al. 1994; Bertauche et al. 1996). A similar analysis of the *Oryza sativa* L. genome revealed that a maximum of 243 proteins in *O. sativa* L. possibly have EF-hand motifs (Boonburapong and Buaboocha 2007). Over 100 of these possible EF-hand proteins also had only one canonical EF-hand. Ca^{2+} binding assays need to be performed to test the actual binding of Ca^{2+} to those proteins predicted to bind Ca^{2+} but not yet verified to be Ca^{2+}-binding.

2.1.2 Families of EF-Hand Proteins

Phylogenetic analysis identified six major groups of EF-hand containing proteins in Arabidopsis within which were several families of proteins (Day et al. 2002). Three families have been extensively characterized, calmodulin (CaM) and calmodulin-like (CML) proteins, Ca^{2+}-dependent protein kinases (CPKs), and calcineurin B-like (CBLs) proteins. CaMs have two pairs of EF hands (Fig. 2a, b) and no other domain, while CaM-like proteins have been defined as proteins composed of EF hands and no other known or identifiable functional domains and that share at least 16% amino acid identity with CaM (McCormack et al. 2005). McCormack et al. (2005) identified seven CaMs and 50 CML proteins in Arabidopsis. Unlike vertebrate CaMs that are 100% identical on the protein level, the seven CaM genes

code for four CaM protein isoforms. Thirty-four CDPKs with four distinct domains, an N-terminal variable domain, a protein kinase domain, an autoinhibitory domain, and a calmodulin-like domain have been identified in the Arabidopsis genome (Fig. 2a) (Cheng et al. 2002; Day et al. 2002). Ten *CBL* genes (Fig. 2a), EF-hand proteins most similar to the regulatory B subunit of calcineurin (a protein phosphatase) and neuronal Ca^{2+} sensors of animals, have been identified either experimentally or from the completed genome sequence (Kolukisaoglu et al. 2004; Kudla et al. 1999; Liu and Zhu 1998). They interact specifically with a group of Ser/Thr protein kinases termed CBL-interacting protein kinases (CIPKs). These major families were recently reviewed by DeFalco et al. (2010). The rice analysis also revealed multimembered families (Boonburapong and Buaboocha 2007). Five calmodulins were identified as well as 32 CaM-like proteins, and a phylogenetic tree shows the presence of a family of CDPKs and a family of CBLs.

A fourth family has also been characterized, the Rboh (respiratory burst oxidase homology) family. The Rboh family has 10 members in Arabidopsis ranging in size from 70 to 108 kd (Day et al. 2002; Keller et al. 1998; Torres et al. 1998). The Rboh genes encode the key enzymatic subunit of the plant NADPH oxidase and the Rboh proteins are the source of reactive oxygen intermediates (ROS) produced following pathogen recognition and in a variety of other processes (Torres and Dangl 2005). The plant Rboh genes have a 300-amino acid amino-terminal extension with two EF-hands that bind Ca^{2+} (Fig. 3a). As in the rice Rboh, OsRbohB, the second EF hand can be noncanonical, not binding Ca^{2+} but acting as a pair with the first EF hand as found in the normal paired EF-hand proteins (Oda et al. 2010). Recently, Ogasawara et al. (2008) showed that ROS production by *Arabidopsis thaliana* rbohD (AtrbohD, At4g47910) was induced by ionomycin, a Ca^{2+} ionophore that induces Ca^{2+} influx into the cell. The activation required a conformational change in the EF-hand region, as a result of Ca^{2+} binding to the EF-hand motifs. They also showed that AtrbohD was directly phosphorylated in vivo suggesting that Ca^{2+} binding and phosphorylation synergistically activate the ROS-producing enzyme activity of AtrbohD.

2.1.3 Other EF-Hand Proteins

Besides these families of proteins, several Ca^{2+} -binding proteins have been reported in the literature. These include CaM-like proteins (CMLs, McCormack et al. 2005) such as CML42 that binds three molecules of Ca^{2+} and functions in trichome development (Dobney et al. 2009) and two CMLs, TCH2 (At5g3770, CML24) and TCH3 (At2g41100, CML12) that were found as touch-induced proteins (Braam and Davis 1990). TCH2 functions in response to abscisic acid, day length, and ion stress (Delk et al. 2005). Centrin1 (CML20, At3g50360) was isolated as being rapidly induced after pathogen inoculation while Centrin2 (CML19, At4g37010) has been shown to be involved in nucleotide excision repair (Cordeiro et al. 1998; Liang et al. 2006). Other CMLS that have been reported in the literature are CML9 that plays a role in salt stress tolerance through ABA

(Magnan et al. 2008), a plasma membrane protein (PM1, At2g41410, CML35) (Bartling et al. 1993), and a Ca^{2+}-binding protein (At5g17480, CML32) in pollen (Rozwadowski et al. 1999).

A Ca^{2+}-signal sensing GTPase (At3g63150) contains a RHO-like GTPase domain at the N-terminus and two Ca^{2+}-binding EF-hand motifs (Fig. 2a) that have the capability to bind Ca^{2+} and its GTPase activity is regulated by changes in Ca^{2+} concentration (Jayasekaran et al. 2006a). Expression was induced by ABA and salt stresses and knockouts were highly sensitive to ABA and salt treatments. A putative EF-hand loop was identified in a glutamate dehydrogenase (GDH2, At5g07440) but not in GDH1 (Turano et al. 1997). At5g54490 was predicted to have one EF-hand (Day et al. 2002). Further analysis of this gene (*PBP1*) revealed that it had one canonical EF hand and two noncanonical EF hands (Benjamins et al. 2003). It had been isolated as a protein that interacts with the protein kinase, PID, and the interaction of PID and PBP1 was shown to be Ca^{2+} dependent (Benjamins et al. 2003). NaCl-inducible gene product AtNIG1 (At5g46830) is a transcription factor that has been shown to bind Ca^{2+} (Kim and Kim 2006). Its one EF-hand domain is noncanonical in not having a G residue at 6. AtNIGI was found to bind the E-box-DNA sequence of promoters of known salt stress genes. AtCP1 (At5g49480), also implicated in salt stress, has three EF hands and has been shown to bind Ca^{2+} (Jang et al. 1998).

A single EF-hand Ca^{2+}-binding protein, KIC (KCBP-interacting Ca^{2+}-binding protein), was isolated as a protein that interacts with the calmodulin-regulated kinesin KCBP (Sect. 4) (Reddy et al. 2004). KIC was shown to bind Ca^{2+}, the interaction with KCBP was Ca^{2+} dependent and the interaction negatively regulates the binding of KCBP to microtubules and the microtubule-dependent ATPase activity of KCBP. Neither centrin (At4g37010) nor the two proteins showing homology to KIC (At4g27280 and At5g54490) interacted with or regulated KCBP. The structure of the KIC/KCBP interaction has been solved (Fig. 2c) (Vinogradova et al. 2009). KIC binds to an amphipathic helix that lies between the neck and a negatively charged region of the C-terminus of KCBP. The binding of Ca^{2+}-bound KIC to the motor functions as an allosteric trap where the neck mimic of KCBP associates with KIC rather than itself resulting in stabilization of the motor in the ADP conformation. The motor is then arrested in a state that has weak affinity for microtubules. The interactions of motor with microtubules are further destabilized by steric hindrance and electrostatic repulsion between the negative coil of KCBP and the negatively charged C-terminus of tubulin. Interestingly, the structure of KIC reveals that there is another EF hand of nearly identical conformation but because of its amino acid composition, the loop of this EF hand does not bind metal ions (Fig. 2a) (Vinogradova et al. 2009). When KIC interacts with KCBP, the canonical hand is loaded with Ca^{2+} but not the noncanonical EF hand.

About half the potential EF-hand proteins in rice were found to have no other domain. The other half had additional domains that gave clues to their functions which in addition to the families mentioned above included transcription factors,

ion channels, DNA- or ATP/GTP-binding proteins, mitochondrial carrier proteins, protein phosphatases, and protein kinases (Boonburapong and Buaboocha 2007).

2.1.4 EF-Hand-Like Sensors

Some proteins were identified in the literature as EF-hand-like Ca^{2+}-binding proteins. These included 14-3-3's, SUBS, and caleosins (Lu et al. 1994; Guo et al. 2001; Naested et al. 2000). Lu et al. (1994) reported that GF14 omega, a 14-3-3 protein, binds Ca^{2+} in the C-terminal domain that contains a sequence bearing homology to the loop of the EF hand (Fig. 3a). However, the surrounding sequence does not form the helix–loop–helix motif and an EF hand is not recognized by SMART (http://smart.embl-heidelberg.de/) or InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). Later reports show that 14-3-3 binds divalent cations including Ca^{2+} and Mg^{2+} (Athwal et al. 1998). SUB1 was found to function as a component of a cryptochrome-signaling pathway and as a modulator of a phytochrome-signaling pathway (Guo et al. 2001). The SUB1 protein was shown to be a Ca^{2+}-binding protein but the EF-hand-like sequence was very diverged from the conserved sequence.

Caleosins were identified as Ca^{2+}-binding proteins that were found associated with lipid bodies (Naested et al. 2000). Recently, based on their peroxygenase activity, caleosins Clo-1 and Clo-2 in Arabidopsis were renamed AtPXG1 and AtPXG2 (Hanano et al. 2006). Although it was reported by Naested et al. (2000) that caleosins have an EF hand, the protein analysis programs such as InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) and SMART (http://smart.embl-heidelberg.de/) did not recognize the EF hand. Close analysis of the sequence of Clo-1 revealed a putative Ca^{2+}-binding loop (Fig. 3a, AtPXG1) that is noncanonical. However, caleosins do bind Ca^{2+} and Ca^{2+} binding does have an effect on their activity (Hanano et al. 2006). Recent work using caleosin isoform 3 (Clo-3) shows that it is upregulated in response to stresses and has the same peroxygenase activity as isoform Clo-1/AtPXG1 (Partridge and Murphy 2009).

2.2 *Non-EF-Hand Ca^{2+} Sensors*

The C2 domain was first characterized in protein kinase C isoforms that are Ca^{2+} dependent (Hug and Sarre 1993). Crystal structural analysis revealed the Ca^{2+}-binding site in the C2 domain of synaptotagmin I and corresponding residues were found in animal and plant C2-containing proteins (Kopka et al. 1998b; Sutton et al. 1995). A synaptotagmin in Arabidopsis, SYTA (At2g20990) regulates endosome recycling and movement and protein-mediated trafficking of plant virus genomes through plasmodesmata (Lewis and Lazarowitz 2010). It is also important for adaptation to salt and cold stress (Schapire et al. 2008; Yamazaki et al. 2008). Other C2 domain proteins in plants include phospholipase

C (PI-PLC, Fig. 3a) – three distinct PI- PLC isoforms, StPLC1, StPLC2, and StPLC3, cloned from potato leaves contain C2-like domains with an optimal PIP2-hydrolyzing activity at 10 μM Ca^{2+} (Kopka et al. 1998a); BAP1, a Ca^{2+}-dependent phospholipid-binding protein involved in negative regulation of defense responses (Yang et al. 2006); a pepper protein (CaSRC2-1) upregulated in response to infection whose C2 domain is necessary for membrane localization (Kim et al. 2008), and OsPBP1 a novel functional C2-domain phospholipid-binding protein that is required for pollen fertility likely by regulating Ca^{2+} and phospholipid-signaling pathways (Yang et al. 2008). Phospholipase D (PLD), which cleaves membrane phospholipids into a soluble head group and PA, also has a C2 domain (Wang and Wang 2001).

Calreticulin is a Ca^{2+}-binding protein localized to the ER that has been extensively characterized in animals and first identified in plants in Ca^{2+}-binding spinach proteins (Menegazzi et al. 1993). Calreticulins have two Ca^{2+}-binding domains, the P domain having high affinity but low capacity and the C domain having high capacity (Jia et al. 2009). Calreticulins function in sequestering Ca^{2+} in the ER and as a molecular chaperone (Jia et al. 2009; Tuteja and Mahajan 2007). A protein expressed in pistils (pistil-expressed Ca^{2+}-binding protein, PCP) was shown to bind Ca^{2+} with a low affinity but high capacity (Furuyama and Dzelzkalns 1999). The Ca^{2+}-binding domain of PCP is similar to the low-affinity/high-capacity domain of calreticulin.

A Ca^{2+}-sensing receptor, CAS, has been localized to the plasma membrane and is expressed predominantly in the shoot, including guard cells (Han et al. 2003). It exhibits low-affinity/high-capacity Ca^{2+} binding in the N-terminal portion of the protein. CAS mediates Ca^{2+} changes in the $[Ca^{2+}]_{cyt}$ due to changes in the Ca^{2+} concentration in the cell wall at the exterior surface of the plasma membrane ($[Ca^{2+}]_o$). It was shown that CAS is involved in $[Ca^{2+}]_o$-induced $[Ca^{2+}]_{cyt}$ increases. Plants deficient in CAS did not exhibit $[Ca^{2+}]_o$-induced $[Ca^{2+}]_{cyt}$ increases and stomatal closing was impaired. Bolting was delayed in CAS-deficient plants indicating a role in the developmental switch from vegetative to reproductive state. Later it was shown that $[Ca^{2+}]_{cyt}$ oscillations are synchronized to $[Ca^{2+}]_o$ mainly through CAS and that CAS regulates the concentration of 1,4,5-triphosphate which is involved in the release of Ca^{2+} from internal stores (Tang et al. 2007).

3 Ca^{2+} Sensor Targets

Some of the EF-hand proteins have other domains that function in signaling pathways by their activity such as the Rboh family; that have an enzymatic function; and are involved in ROS production (Keller et al. 1998; Torres et al. 1998). The action of these proteins produces products involved in Ca^{2+} signaling. A few Ca^{2+} sensors have been shown to bind promoters and regulate gene expression. An example of a nonprotein target of a Ca^{2+} sensor is AtNIG1, a transcription factor, whose target is the promoter of stress-induced proteins (Kim and Kim 2006).

AtCaM7 has also been shown to interact with DNA, in this case with a Z-box light responsive element (LRE) in the promoters of *CAB1* and *RBCS1A* (Kushwaha et al. 2008). However, a majority of Ca^{2+} sensor targets identified to date are proteins. The Ca^{2+}-dependent protein kinases as their name implies interact with other proteins and phosphorylate them (Harper et al. 2004; Harper and Harmon 2005). The chapter on Ca^{2+}-dependent protein kinases (CDPKs) of this volume and the review by DeFalco et al. (2010) deal with the targets of these Ca^{2+} sensors.

Many of the EF-hand Ca^{2+} sensors have no other known functional domain and Ca^{2+} signaling by these sensors depends on their ability to bind other proteins, which are in turn activated or repressed by this interaction. Some of the sensor targets have been identified in yeast two-hybrid assays such as PBPI and TCH3 (Benjamins et al. 2003). A yeast-two hybrid screen with a non-Ca^{2+}-binding protein kinase, PINOID, resulted in the isolation of binding partners PBPI, an EF-hand Ca^{2+}-binding protein that had not previously been reported in the literature, and TCH3, a known Ca^{2+}-binding EF-hand protein. An in vitro pull-down assay identified a target of Centrin2 (Liang et al. 2006). A protein involved in nucleotide excision repair, AtRAD4 was shown to bind to Centrin2 and it was shown that the C-terminal EF-hand-containing domain was critical to the interaction (Liang et al. 2006). The CBL family of Ca^{2+} sensors interacts with a family of protein kinases termed CBL-interacting protein kinases (CIPKs) (Weinl and Kudla 2009). See chapter "The CBL–CIPK Network for Decoding Calcium Signals in Plants" of this volume and (DeFalco et al. 2010) for a discussion of CBLs and CIPKs.

CaMs when activated by Ca^{2+} binding interact with many different protein targets. CaM-binding proteins act in a variety of processes including plant defense. In tobacco, 13 CaM genes were found to be regulated transcriptionally and posttranscriptionally in response to wounding and tobacco mosaic virus infection (Yamakawa et al. 2001). Constitutive expression of soybean CaMs, SCaM4 and SCaM5 results in spontaneous leaf lesions and the induction of expression of several defense-related genes (Heo et al. 1999). Two studies link NO generation to $[Ca^{2+}]_{cyt}$ elevation and the involvement of CaM or a CML (Choi et al. 2009; Ma et al. 2008). A CaM-like protein in tobacco, termed rgs-CaM, was isolated in a yeast two-hybrid screen using HC-Pro, a viral protein involved in gene silencing repression (Anandalakshmi et al. 2000). The rgs-CaM itself suppresses gene silencing. Many targets have been identified using CaM as a probe to screen expression libraries (Reddy et al. 2002). Chapter the Calmodulin and several reviews (Kim et al. 2009; Zhu et al. 2001; DeFalco et al. 2010; Boursiac and Harper 2007; Reddy and Reddy 2004b) deal with these Ca^{2+} sensor targets. Three CaM-binding proteins isolated in our laboratory will be discussed in the following sections.

3.1 Kinesin-Like Calmodulin-Binding Protein

Kinesin-Like Calmodulin-Binding Protein (KCBP), a microtubule motor protein, was identified from an expression library using labeled calmodulin as a probe

(Reddy et al. 1996a, b). The three CaM isoforms 2, 4, and 6 can bind KCBP but the binding affinity of CaM2 is two times greater than the binding affinity of CaM4 and 6 (Reddy et al. 1999). Using microtubule binding assays, it was shown that CaM/Ca^{2+} binding resulted in KCBP release from microtubules (Narasimhulu et al. 1997). KCBP movement on microtubules was found to be negatively regulated by Ca^{2+}/CaM as was its microtubule-stimulated ATPase activity and KCBP was also shown to bundle microtubules that are dissociated in a Ca^{2+}/CaM-dependent manner (Song et al. 1997; Deavours et al. 1998; Kao et al. 2000). Structural analysis showed that CaM binds to the predicted CaM-binding helix and inserts itself between the motor and the microtubule (Vinogradova et al. 2004, 2008). KIC, as discussed in Sect. 2.1.3, binds to the same helix as CaM. CaM and KIC, however, have different affinities for Ca^{2+} and so different Ca^{2+} levels may affect which sensor binds to KCBP (Reddy et al. 2004). In vitro immunofluorescence microscopy using affinity-purified anti-KCBP antibody showed that KCBP localizes to the preprophase band, the mitotic spindle, and the phragmoplast (Bowser and Reddy 1997), and studies in Haemanthus endosperm showed localization to anaphase spindle poles (Smirnova et al. 1998). KCBP also has a role in trichome development (Reddy and Day 2000). The wild-type gene of a trichome development mutant (*zwi*) was identified as KCBP (Oppenheimer et al. 1997). Wild-type trichomes have three branches while the *zwi* mutant trichomes have a short stalk with one or two branches depending on the severity of the allele (Oppenheimer et al. 1997). Analysis of the genomes of plants and animals showed that *KCBP* was conserved in plant genomes and was not present in animal genomes although a sea urchin kinesin also contains a calmodulin-binding domain but shows very little homology to KCBP and lacks several conserved domains in KCBP (Abdel-Ghany and Reddy 2000).

3.2 Pollen-Specific Calmodulin-Binding Protein

A maize pollen calmodulin-binding protein (MPCBP) was isolated in a protein–protein interaction-based screening using ^{35}S-labeled CaM as a probe (Safadi et al. 2000). MPCBP contains three tetratricopeptide repeats (TPR) and shares a high sequence identity with three hypothetical TPR-containing proteins from Arabidopsis. MPCBP binds bovine CaM and three CaM isoforms from Arabidopsis in a Ca^{2+}-dependent manner and this binding was mapped to an 18-amino acid stretch between the first and second TPR regions (Safadi et al. 2000). Western, Northern, and RT-PCR analysis have shown that MPCBP expression is specific to pollen and is present in mature and germinating pollen. The three Arabidopsis genes showing similarity to MPCBP were cloned from Arabidopsis pollen (Golovkin and Reddy 2003). One of the proteins, NPG1 (no pollen germination, At2g43040), is expressed only in pollen, whereas the NPG-related proteins (NPGR1, At1g27460 and NPGR2, At4g28600) are expressed in pollen and other tissues. Bacterially expressed NPG1 binds three isoforms of Arabidopsis CaM in a Ca^{2+}-dependent manner as did the putative CaM-binding domain of NPG1. NPGR1

and NPGR2 have conserved CaM-binding domains and also interact with CaM (unpublished data). NPGR1 has been shown to be involved in pollen germination; as shown in a *npgr1*/*quartet* double mutant, pollen containing the *npgr1* mutation do not germinate (Golovkin and Reddy 2003).

3.3 *AtSR1/EICBP1/CAMTA3 Is a CaM-Binding Transcription Factor*

AtSR1/EICBP1/Camta3 (AtSR1, forthwith) was isolated in a CaM-binding screen of a library from ethylene-treated Arabidopsis seedlings (Reddy et al. 2000; Yang and Poovaiah 2002). It is a member of a class of Ca^{2+}/CaM-binding transcription factors also termed CAMTAs (Bouche et al. 2002; Yang and Poovaiah 2002). T-DNA mutants of AtSR1 showed constitutive disease resistance and elevated levels of salicylic acid suggesting that AtSR1 is a negative regulator of plant immunity (Du et al. 2009; Galon et al. 2008). At 19–21°C, *Atsr1-1* plants showed reduced growth, and the expression of resistance-associated marker genes, PR1, PR2, and PR5, was constitutively activated under low temperature. Disease resistance in the mutant was also enhanced in comparison with plants grown under higher temperature. AtSR1 binds to the promoter of EDS1, one of the key enzymes in salicylic acid biosynthesis, and represses its expression. In the mutant, EDS1 is derepressed resulting in SA accumulation at low temperature. Interestingly, AtSR1/CAMTA3 was also found to be a positive regulator of cold-induced gene expression (Doherty et al. 2009). Known cold-induced genes *CBF2, CBF1*, and *ZT12* were downregulated as well as downstream targets of CBF transcription factors, suggesting that it can function as both a positive and a negative regulator of gene expression. For more details on CAMTAs, see chapter on CAMPTAs. In addition to AtSR1, other CBPs (e.g. CNGSs, PICPBs, CBP60s) are involved in plant defense responses (Ali et al. 2003, 2007; Reddy et al. 2003; Wang et al. 2009; Urquhart et al. 2007; Ma et al. 2009).

4 Proteomic Approaches to Elucidating the Ca^{2+} Signaling Components and Networks

4.1 *High-Throughput Methods*

The ability to bind Ca^{2+} by the predicted 250 EF-hand proteins in Arabidopsis has only been shown in comparatively few proteins. In some cases, actual Ca^{2+} binding was shown and in others a Ca^{2+}-dependent activity or interaction. For some of the proteins Ca^{2+} binding was assumed because of their homology to proteins found in animals or other species of plant that had been shown to bind Ca^{2+}. Evaluating the Ca^{2+}-binding status, and therefore the involvement in Ca^{2+} signaling, of each

predicted Ca^{2+}-binding protein could be a labor-intensive process. Similarly, only some of the targets of Ca^{2+} sensors have been identified. However, many more targets have yet to be identified and identifying the Ca^{2+} sensor targets, again, would be a labor-intensive process. Therefore, high-throughput approaches are being developed to study the Ca^{2+} binding of predicted Ca^{2+} sensors and the interacting partners of the sensors.

4.1.1 Protein Arrays

Protein arrays are a high-throughput method that could be used to determine Ca^{2+} binding and Ca^{2+} sensor binding to targets (Fig. 4). As a proof of concept for this, we obtained the clones of a number of known and predicted EF-hand Ca^{2+}-binding proteins and transferred them to expression vectors. Bacterially expressed proteins were purified, electrophoresed, transferred to blots, and tested for Ca^{2+} binding; 29 of the tested proteins showed Ca^{2+} binding at physiological Ca^{2+} concentrations (Day, Brauch, Reddy, unpublished results). To look at the specificity of Ca^{2+} sensor/target interaction, purified EF-hand proteins were also tested for their Ca^{2+}-regulated binding to KCBP, a known target of CaM (Reddy et al. 1996b). KCBP specifically bound to the CaMs and a known KCBP-interacting protein KIC (Reddy et al. 2004). If a protein chip for all predicted EF-hand proteins or a whole

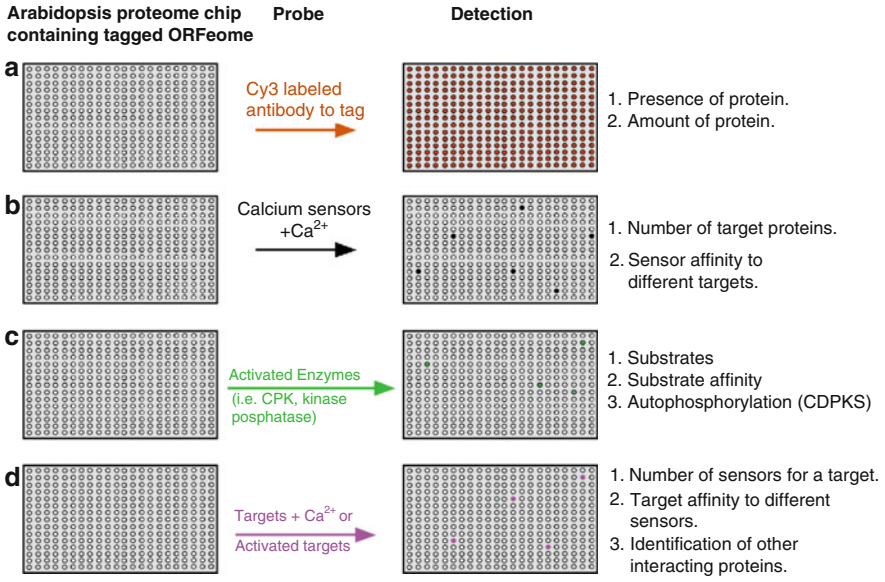

Fig. 4 Protein arrays to analyze interaction network of Ca^{2+}-signaling protein. (**a**) Proteins on the chip can be probed with an antibody to the tag to determine the presence and amount of protein. (**b–d**) Different assays that could be used for elucidating the network of Ca^{2+}-signaling components

proteome chip was available, the proteins on the chips could be easily tested for Ca^{2+} binding and sensor affinity to Ca^{2+}. Additionally, a whole proteome chip could be probed with Ca^{2+} to identify unknown non-EF-hand proteins that bind Ca^{2+}.

Protein chips could be a powerful tool for identifying Ca^{2+} sensor targets. A yeast protein chip containing 5800 ORFs screened with a labeled CaM resulted in the identification of 39 CaM-binding proteins (Zhu et al. 2001). Partial protein microarrays for Arabidopsis have been constructed and used for determining targets of CaM. An array containing 1,133 Arabidopsis proteins was screened with several CaMs/CMLs (Popescu et al. 2007). Previously known and novel CaM/CML targets were identified. These included receptor and intracellular protein kinase transcription factors, RNA-binding proteins, F-box proteins, and proteins of unknown function. Showing the complexity of the Ca/CaM signaling network, multiple CaM/CML proteins had many binding partners while some were specific to one or a few CaMs/CMLs (Popescu et al. 2007). Using protein chips and other labeled Ca^{2+} sensors, the targets of the sensor could be elucidated (Fig. 4). The flip side of this would be to use either a chip with known Ca^{2+} sensors or whole proteome proteins and test the targets to see if they interact with multiple sensors.

Targets of Ca^{2+} sensors that are enzymatic in nature such as the CDPKs can also be identified using protein chip technology. A semidegenerate peptide array containing Ser and Thr residues was used to identify targets of a CDPK along with three other kinases (Vlad et al. 2008). The peptides on the arrays were phosphorylated by the bacterially expressed protein kinases and the phosphorylated peptides for each kinase were analyzed. Peptides that were preferred for each kinase were used for searching protein databases and predicted targets of the kinases were found. While this did not positively identify targets of the CDPK, it suggested possible targets that could be tested for interaction. Protein chips containing 4,400 proteins from yeast were used for finding substrate targets of yeast kinases (Ptacek et al. 2005). Eighty-two kinases were incubated with the chips and phosphorylated proteins on the chip were identified. This would be a method that could be used to identify phosphorylation targets of the CDPKs or CIPKs (Fig. 4).

4.1.2 Other High-Throughput Techniques

High-throughput yeast two-hybrid assays that are semiautomated can be used to screen large numbers of possible interacting proteins. Two CDPKs (AtCDPK4 and AtCDPK11) were used as bait to screen two expression libraries of over 18 million prey clones each (Franzosa et al. 2009). The CDPKs were expressed in a constitutionally active form resulting in the identification of more interacting proteins than in screens using the native form of the CDPKs. The assay system used computerized tracking of samples to perform large-scale transformations and mating reactions in 96-well plates (Franzosa et al. 2009). Using a framework that uses an empirically based approach to rigorously dissect quality parameters of currently available human interactome maps, Venkatesan et al. (2009) found that high-throughput yeast two-hybrid interactions for human proteins are more precise

than literature-curated interactions supported by a single publication, suggesting that HT-Y2H is suitable to map a significant portion of the human interactome.

CaM-binding proteins in the human proteome and *C. elegans* were identified using the mRNA display technique (Shen et al. 2005, 2008). In this technique, a cDNA library is transcribed and the mRNA is conjugated to puromycin. The conjugated mRNA is translated and the protein is fused to the mRNA through the puromycin moiety (Kurz et al. 2000, 2001). The mRNA is reverse transcribed and purified based on tags in the fusion. The DNA/protein fusions are incubated with biotinylated Ca^{2+}/CaM and passed through a strepavidin column to bind the biotinylated CaM. Eluted DNA/protein fusions are PCR amplified for another round of screening or sequencing for identification of the genes and thus protein products of the genes. This could be a powerful method to discover the targets of not only CaM but other Ca^{2+} sensors also.

4.2 Tandem Affinity Purification

It is likely that the proteins involved in a Ca^{2+}-signaling pathway interact in complexes. Interaction assays such as yeast two-hybrid and protein chip hybridization reveal only direct protein-to-protein interaction. In order to identify other interacting proteins in a complex, in vivo complexes must be isolated. A method to isolate complexes has been developed and used in yeast (Gavin et al. 2002), insect (Forler et al. 2003), and plant systems (Rohila et al. 2004, 2006; Rubio et al. 2005; Van Leene et al. 2007)

Tandem affinity purification (TAP) is an MS-based approach for identifying interacting proteins that form complexes. A gene for a target protein is fused to a tandem affinity protein tag (TAP-tag), a host organism is transformed with the tag, and the TAP-tagged protein and any associated proteins are then isolated from the host organism in two sequential affinity purifications. The two affinity tags are separated by a TEV protease site and are attached to the N- or C-terminus of the protein of interest. This TAP cassette is introduced into the living system and expressed at a level equal to its endogenous level. Then the cell extracts are processed and purified using affinity beads for the first tag and the bound proteins are eluted with TEV protease. The eluted protein complex is then passed through a second affinity matrix and proteins from the second elution are treated with trypsin. Masses of resultant peptides are determined using MALDI-TOF mass spectrometry and/or (LC) nano-ESI-MS/MS and the peptide mass list is used to search against protein databases to identify proteins. Modifications of the method have adapted this system for use in plants (Rohila et al. 2004). Protein kinase interactions in rice have been studied by this method (Rohila et al. 2006, 2009). Protein kinases fused to the tandem tags were expressed in rice and TAP purification identified interacting proteins for many of the protein kinases. Van Leene et al. (2007) studied the cell cycle interactome in Arabidopsis and 28 new molecular associations were revealed

as well as confirmation of 14 previously described interactions. They concluded that this systemic approach is generally applicable to other pathways in plants.

4.3 Functional Analysis of Ca^{2+}-Signaling Components

The proteins expressed in plant cells vary with cell type, developmental stage, and biotic and abiotic stimulation. Analyses of proteins present at a given time point or under given conditions provide more evidence of the function of a protein. Localization of the sensors spatially and temporally can give information about function. Loss-of-function mutants or plants overexpressing a protein may have phenotypes that give evidence of its function.

4.3.1 Fluorescent Labeling of Protein

Fusion of green fluorescent protein (GFP) or its variants with entire proteins of known or unknown function can show where proteins are located, whether the proteins move from one compartment to another, or whether two proteins associate (Berg and Beachy 2008; Hanson and Kohler 2001). Fusion constructs can be used for transient expression or *Agrobacterium*-mediated plant cell transformation. GFP and variants with different spectral properties can be targeted to separate compartments to determine their location, mobility, and dynamic changes during development, environmental response, or Ca^{2+} influx using ionophores (Hanson and Kohler 2001; Berg and Beachy 2008).

Using the single-celled pollen tube, the expression of several Ca^{2+} sensors was studied, including CaM, CMLs, a CDPK, and a CBL (Zhou et al. 2009). Pollen was transformed with the genes fused to GFP under the strong pollen-specific LAT52 promoter. In another study using CaM-GFP expressed using a pollen-specific promoter, fluorescence was detected at the germinal pores and the tip-to-base gradient of fluorescence was observed in developing pollen tubes (Shi et al. 2009). Accumulation of CaM at the tip resulted in pollen tube growth, but when the distribution of CaM was equal throughout the tip, pollen tube growth stopped. These studies demonstrated the involvement of CaM in pollen tube growth.

Two Ca^{2+}-dependent protein kinases (CPKs), CPK17 and CPK34, were fused to yellow fluorescent protein (YFP) and expressed in pollen (Myers et al. 2009). Both isoforms appear to target to the plasma membrane, as shown by imaging of CPK17-YFP and CPK34-YFP in growing pollen tubes. CPK30 is highly expressed in the root and induced by ABA, IAA, 2,4-D, GA(3), and 6-BA treatments (Yuan et al. 2007). GFP-CPK30 fusion protein was localized to both the cell wall and plasma membrane, suggesting its function as a receptor.

Bimolecular fluorescence complementation (BiFC) is based on the ability of two nonfluorescing fragments of a fluorescent protein (commonly YFP) to complement each other in trans if they are fused to two interacting proteins resulting in the

fluorescence of complemented fragments (Weinthal and Tzfira 2009). Using BiFC, the components of Arabidopsis Ca^{2+}/H^{+} exchangers AtCAX1 and AtCAX3 were studied (Zhao et al. 2009). They demonstrated that two separately expressed halves of CAX1 can interact and, furthermore, demonstrated that a heteromeric interaction could occur between the first half of CAX1 and the C-terminal part of CAX3. Waadt et al. (2008) developed improved BIFC vectors that allow multicolor fluorescence allowing for studying two or more interactions in one cell. With this system they showed the concurrent interaction of the protein kinase CIPK24 with the Ca^{2+} sensors CBL1 and CBL10 at the plasma membrane and tonoplast, respectively, and the simultaneous formation of CBL1/CIPK1 and CBL9/CIPK1 protein complexes at the plasma membrane (Waadt et al. 2008). Using BiFC, CBL3 was shown to interact not only with a CIPK but also with 5′-methylthioadenosine nucleosidase (AtMTAN) (Oh et al. 2008).

4.3.2 Overexpression or Loss-of-Function

Overexpression of KIC in Arabidopsis resulted in trichomes with a reduced branch number resembling the *zwichel*/*kcbp* phenotype. These results suggest that KIC modulates the activity of KCBP in response to changes in cytosolic Ca^{2+} and regulates trichome morphogenesis (Reddy et al. 2004). In the pollen studies using fluorescent-labeled Ca^{2+} sensors (Sect. 4.3.1) done by Zhou et al. (2009), aberrant phenotypes were observed from overexpression of the proteins. CDPK32-GFP caused severe growth depolarization and overexpression of CBL2-GFP and CBL3-GFP inhibited pollen tube elongation and induced growth depolarization. Overexpression of two CDPKs in *Petunia inflata*, PiCDPK1 and PiCDPK2, showed that they are also involved in pollen development (Yoon et al. 2006). Overexpression of *PiCDPK1* disrupted pollen polarity while overexpression of *PiCDPK2* inhibited pollen tube elongation and both mutants had elevated $[Ca^{2+}]_{cyt}$.

T-DNA insertion and antisense, viral and RNAi silencing can be used to dissect the roles of Ca^{2+} channels, sensors, or sensor targets. A pollen receptor-like kinase LePRK2 localized in the plasma membrane of tomato (*Solanum lycopersicum*) pollen tubes has been implicated in having a signaling role during pollen germination and tube growth (Zhang et al. 2008). Using transgenic tomato plants transformed with a construct including a full-length LePRK2 antisense DNA driven by a pollen-specific promoter (LAT52), Zhang et al. (2008) showed that antisense LePRK2 pollen tubes do not show increased growth upon addition of Ca^{2+} ions whereas wild-type plants do. These results along with other evidence suggest that LePRK2 is involved in transducing the extracellular Ca^{2+} signal.

The role of two RBOHs from *Nicotiana benthamiana*, NbrbohA and NbrbohB that encode plant NADPH oxidase, was studied using virus-induced gene silencing (Zhang et al. 2009). Their results indicated that *N. benthamiana* RBOHs function in elicitor-induced stomatal closure, but not in elicitor-induced HR. SYT1 (At2g20990) is a homologue of synaptotagmin, a Ca^{2+} sensor that initiates exocytosis (Kawamura and Uemura 2003). In a leaf-freezing experiment

Ca^{2+}-dependent freezing tolerance was inhibited by the extracellular addition of an antibody against the cytosolic region of SYT1 (Yamazaki et al. 2008). Using SYT1-RNAi, protoplasts isolated from Arabidopsis lost Ca^{2+}-dependent freezing tolerance and intact SYT1-RNAi plants had lower freezing tolerance than control plants.

T-DNA insertion mutants have been used to study the function of many proteins involved in Ca^{2+} signaling, a few examples follow. KCBP/ZWICHEL (Sect. 3.1), isolated as a T-DNA insertion mutant with an aberrant trichome phenotype, was found to be a CaM/Ca^{2+}-regulated kinase (Oppenheimer et al. 1997). A double disruption of CPK17 and CPK35 using T-DNA insertion resulted in an approximately 350-fold reduction in pollen transmission efficiency (Myers et al. 2009). The phenotype of the double mutants could be rescued through pollen expression of a CPK34-YFP fusion while a transgene rescue was blocked by mutations engineered to disrupt the Ca^{2+} activation mechanism of CPK34, providing in vivo evidence linking Ca^{2+} activation to a biological function of a CPK. A T-DNA mutant of CIPK8, a CBL-interacting protein kinase gene, showed that CIPK8 positively regulates the nitrate-induced expression of primary nitrate response genes, including nitrate transporter genes and genes required for assimilation (Hu et al. 2009). Seed germination of *cml9* T-DNA insertion mutants was more sensitive to salt and the sensitivity was mediated by ABA (Magnan et al. 2008). The expression of known stress-responsive genes (RAB18, RD29A, RD20 and ERD10) was also studied in the *cml9* mutants. Delayed and weaker induction was shown for these genes in the *cml9* mutants and a transient rather than a sustained expression of RAB18, RD29A, and RD20 genes. Gain-of-function, knockout (double or triple mutants in the case of families), dsRNAi, and artificial miRNA are powerful tools to decipher biological roles of any known or unknown protein even if it is a member of a family.

5 Concluding Remarks

Many proteins that are involved in Ca^{2+} signaling have been identified and several have been characterized. However, much more work remains to be done. The molecular and phenotypic information gathered from the above methods can be used to advance our understanding of the Ca^{2+}-signaling network. Proteomic information can be compiled and analyzed on a large scale. (See Baerenfaller et al. 2008; Jorrin-Novo 2009; Baginsky et al. 2010 for reviews of the methods, shortcomings, and strengths of proteomic analysis.) As more protein arrays become available and more data from protein array studies, yeast two-hybrid assays and other high-throughput methods, a network of interacting proteins can be constructed and critical hubs in the network can be identified that will help in elucidation of Ca^{2+}-signaling pathways. Techniques are available for construction of interactomes using data generated and mined from databases (Popescu et al. 2007; Franzosa et al. 2009; Huang and Fraenkel 2009). The challenge for

researchers is to obtain the proteomic data and construct networks to get a global view of Ca^{2+}-signaling pathways.

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Decoding of Calcium Signal Through Calmodulin: Calmodulin-Binding Proteins in Plants

Liqun Du, Tianbao Yang, Sathyanarayanan V. Puthanveettil, and B.W. Poovaiah

Abstract Calmodulin is the best characterized calcium sensor which is highly conserved in all eukaryotes including plants. The flexible structural features enable calmodulin to bind and regulate a broad spectrum of target proteins which are involved in diverse cellular/physiological functions in plants. Hence, calmodulin is considered to be a multifunctional regulatory protein although calmodulin itself usually has no enzymatic or biochemical functions. Plants have multiple calmodulins and calmodulin-like proteins, therefore the Ca^{2+}/CaM-mediated signaling system in plants is considerably more complex as compared to that in animals. In this chapter we describe the structure–function relationships of calmodulin, action models of calmodulin-mediated regulation of target proteins, complexity of calmodulin-mediated regulations, and functional implications of calmodulin and target interactions in plants. Emphasis is placed on functional significance of Ca^{2+}/CaM-mediated regulation of targets with various enzymatic/biochemical/biophysical functions during plant growth and development, and plant response to abiotic and biotic stimuli.

1 Introduction

Calmodulin is a ubiquitous calcium sensor in eukaryotic cells. The concept that calcium-binding proteins could transduce a calcium signal into a physiological response was first proposed by Ebashi and Kodama (1965). They demonstrated

L. Du • B.W. Poovaiah (✉)
Department of Horticulture, Washington State University, Pullman, WA 99164-6414, USA
e-mail: poovaiah@wsu.edu

T. Yang
USDA-ARS Food Quality Laboratory, Beltsville, MD 20705, USA

S.V. Puthanveettil
Columbia University, New York, NY, USA

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that troponin C, a calcium-binding subunit of the troponin complex, exhibited calcium sensitivity to the contractile structure of skeletal muscle cells. A few years later, Cheung and Karkiuchi independently discovered that an activator of cyclic nucleotide phosphodiesterase involved in the regulation of cAMP concentration was stimulated by calcium (Kakiuchi and Yamazaki 1970; Cheung 1971). The activator was found to bind calcium and was similar to troponin C. Eventually named "calmodulin," an abbreviation of *cal*cium-*modul*ated prote*in*, the protein was found to be present in all eukaryotes. Since its discovery about 40 years ago, calmodulin (CaM) has been regarded as a model calcium-binding protein and subjected to intensive studies in biochemistry, cell biology, and molecular biology because of its importance in almost all aspects of cell activity (Poovaiah and Reddy 1987, 1993; Zielinski 1998; Chin and Means 2000; Reddy 2001a; Snedden and Fromm 2001; Yang and Poovaiah 2003; Bouche et al. 2005; Yang et al. 2007; Kim et al. 2009b). In fact, disruption or depletion of the single copy of calmodulin gene in yeast results in a recessive lethal mutation (Davis et al. 1986), suggesting that calmodulin is a house-keeping gene in eukaryotic cells.

The calmodulin sequence was reported in bovine (Watterson et al. 1980). Thus, the vertebrate calmodulins are often regarded as the prototype of calmodulin. The prototype vertebrate calmodulin is a small acidic protein with 148 amino acid residues, a molecular weight of ~16.7 kDa and pI of 3.9–4.3. However, many variations of calmodulin (isoforms) have been reported since plant calmodulins were first characterized (Snedden and Fromm 2001; Yang and Poovaiah 2003). In contrast to animals which have one or a few genes encoding the same calmodulin isoform, plants have multiple genes encoding more diversified isoforms. Nevertheless, the calmodulin family has a remarkable similarity in amino acid sequences among plant and animal kingdoms. Regardless of their sources, all calmodulins are heat stable, monomeric globular proteins with a characteristic dumb-bell shape.

It is difficult to crystallize apo-calmodulin, the calcium-free calmodulin, likely due to the high dynamic nature of the protein. High resolution NMR studies indicate that apo-calmodulin is not dumb-bell shaped as is calcium-loaded calmodulin (Zhang et al. 1995). Instead, the four helices in each domain of apo-calmodulin are arranged in somewhat parallel/antiparallel orientations to one another, giving each domain a relatively compact structure but leaving the calcium-binding loops exposed for easy calcium access (Fig. 1a). However, a calcium-loaded mammalian calmodulin crystal structure was reported in 1985 (Babu et al. 1985), and a further refined structure was reported later using X-ray crystallography and NMR spectroscopy (Babu et al. 1988; Chattopadhyaya et al. 1992; Meador et al. 1993, 1995; Meador and Quiocho 2002). Plant calmodulin structure has been inferred from animal calmodulin crystal structure. In 2004, the three-dimensional structure of the first plant calmodulin, potato PCM6, was reported (Yun et al. 2004). PCM6 is a conserved calmodulin isoform in plants (Takezawa et al. 1995). Like animal calmodulins, PCM6 carries four helix–loop–helix calcium-binding motifs commonly known as EF hands (Fig. 1b, c). The four EF hands are equally distributed in N-terminal and C-terminal globular domains or lobes and connected by an alpha-helix link in the central region. Although the structures of EF hands are very similar,

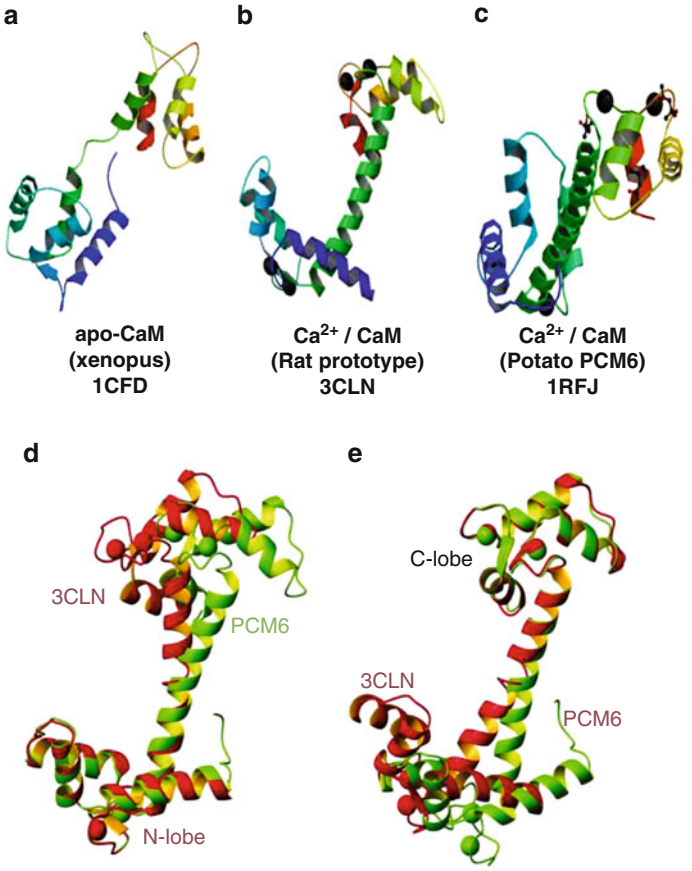

Fig. 1 Three-dimensional structure of calmodulin. Calcium ions are in *black* (**a**, **b**, **c**), and *red* or *green* (**d**, **e**). Structure data are taken from Protein Data Bank (PDB). (**a**) apo-CaM (PDB: 1CFD); (**b**) Rat Ca^{2+}/CaM, a prototype CaM (PDB: 3CLN); (**c**) Potato PCM6 (PDB: 1RFJ); (**d**, **e**) Stereoview showing the superposition of potato camodulin PCM6 (*green*) and rat calmodulin (*red*). (**d**) Superposition of the N-lobes; (**e**) Superposiiton of the C-lobes. Parts (**d**) and (**e**) are adapted from Yun et al. (2004)

there is a major difference in the central helix region possibly due to the different packing arrangement between PCM6 and animal calmodulins. This could be the result of a few amino acid residue substitutions in the intermolecular interaction surface and a number of changes of methionine residues in the hydrophobic patches. It is believed that the four EF hands can sense the calcium changes triggered by external and internal signals and undergo the conformational changes leading to the exposure of the hydrophobic region in the central link and that the hydrophobic pockets in the N-terminal and C-terminal lobes are responsible for molecular recognition of various downstream targets. Therefore, while plant and animal calmodulins can recognize some target proteins with the same binding

motifs, it is reasonable to infer that, considering the many diversified calmodulin isoforms in plants, plant calmodulins can interact with different and much larger number of downstream targets.

Calmodulin's role relies on its ability to physically bind a motif with a stretch of 16–35 residues in the target proteins, once it is loaded with calcium (Rhoads and Friedberg 1997; Hoeflich and Ikura 2002). However, the motif can be as short as 12 residues (Ishida et al. 2009) and as long as 67 residues (Levy et al. 2005). It has been proposed that the recognition motifs for calcium-loaded calmodulin belong to two groups: 1-8-14 and 1-5-10 based on the position of conserved hydrophobic residues. This was inferred from calcium-independent calmodulin-binding IQ motifs (Xie et al. 1994). While many calmodulin-binding motifs fit these rules, some of the recently defined plant calmodulin-binding motifs do not necessarily have these features. However, the target peptides usually form a basic amphipathic helix, which contains hydrophobic residues on one side and basic residues on the other (Fig. 2a). There is often a residue with a large hydrophobic portion of the target peptide, holding the large hydrophobic side chain of the peptide in the hydrophobic pocket of calmodulin to anchor the target peptide, and the acidic clusters of calmodulin then interact with the basic portion of the target peptide. The remarkable flexibility of the central linker and exceptionally large number of methionine residues in the hydrophobic pocket give calmodulin conformational plasticity to adjust to a variety of target peptides (Ikura and Ames 2006). These target peptides are usually intrinsically disordered and likely undergo binding and folding (Radivojac et al. 2006). However, there is not much homology in the amino acid sequence among all the calmodulin-binding motifs. Therefore, in order to understand calmodulin-regulated processes, it is critical to systematically identify calmodulin targets. In particular, since plants have multiple calmodulin isoforms, it is important to understand whether these different proteins operate through the same or different targets. Traditional approaches, such as yeast two-hybrid assays, expression library screening, and SDS/PAGE overlays with labeled calmodulin, have been used to identify calmodulin-binding proteins in plant and animal systems. In particular, using the labeled calmodulin to screen the cDNA expression library has been a very effective approach for isolating plant calmodulin-binding proteins. Most characterized plant calmodulin-binding proteins were isolated using this approach. Recently, a protein microarray approach has been developed, in which the targets of several calmodulin isoforms can be defined based on their specificity of interactions with different partners (Popescu et al. 2007). As the number of protein collections in the protein microarray increase, the approach could be very useful to find the targets for different calmodulin isoforms in an entire genome. So far, about 80 plant calmodulin targets have been characterized. However, it is believed that there are many more putative calmodulin-target proteins yet to be discovered. The current estimate of calmodulin targets in the *Arabidopsis* genome is about 500. These target proteins are involved in almost all aspects of plant growth and development, as well as responses to biotic and abiotic stresses. Table 1 summarizes the characterized calmodulin-binding proteins and their calmodulin-binding motifs.

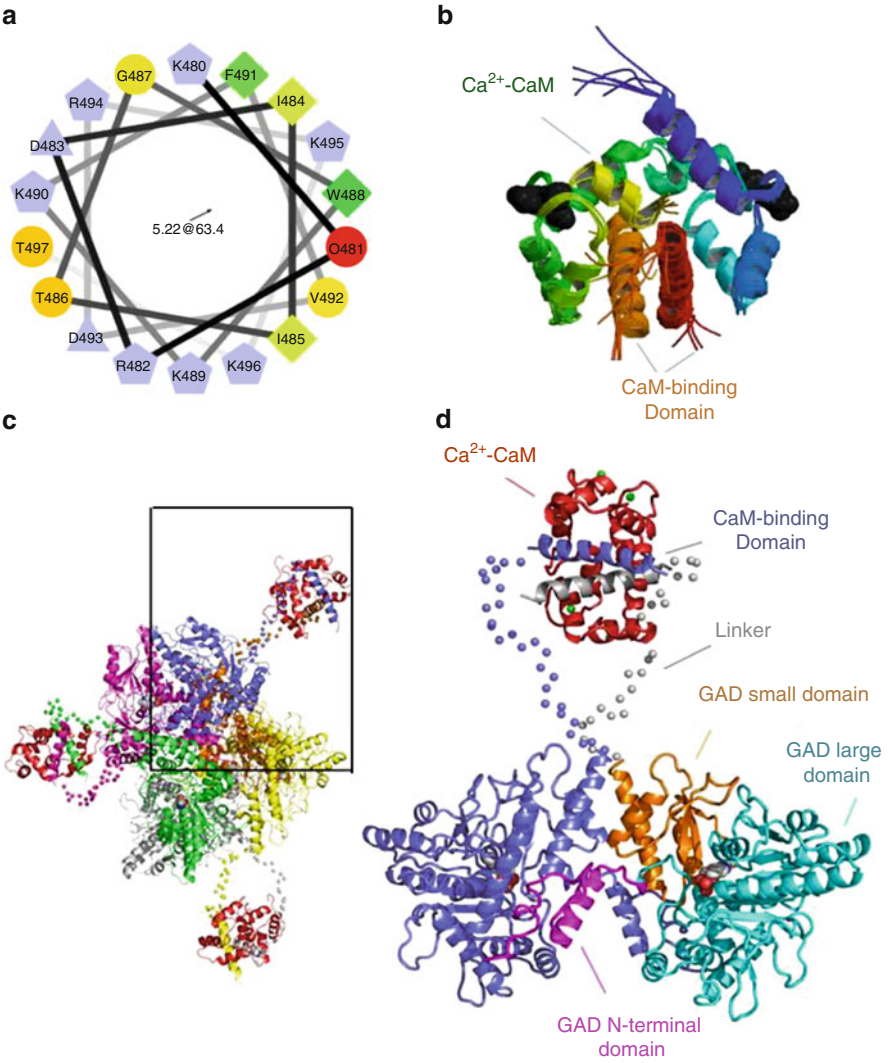

Fig. 2 Ribbon presentation of the structure of calcium–calmodulin–glutamate decarboxylase (GAD). (**a**) Helicalwheel projection of calmodulin-binding domain in *Arabidopsis* GAD1 showing a basic amphipathic alpha helix structure. (**b**) One calcium/calmodulin complex binds two peptides corresponding to the GAD1 CaM-binding domain (PDB:1NWD). (**c**) Three calcium–CaM complexes bind to a hexamer GAD1 complex. Each calcium–calmodulin binds to a pair of CaM-binding domains from nearby two GAD1 subunits. (**d**) Enlarged picture from the box of Fig. 3c showing that one calcium–calmodulin binds to two calmodulin-binding domains to relieve the autoinhibition of GAD catalytic activity. Parts (**c**) and (**d**) are adapted from Gut et al. (2009)

Calmodulin can modulate the actions of target proteins by physically binding to them. Several models have been proposed based on the three-dimensional structural studies of the interactions of calmodulin and the calmodulin-binding proteins/

Table 1 Plant calmodulin-binding proteins with defined calmodulin-binding domain

Protein	CaM binding domain (CaMBD)	*Arabidopsis*	Nonplant system[a]	References
Glutamate decarboxylase	VHKKTDSEVQLEMITAWKKFVEEKKKKTNRVC (Petunia) VKKSDIDKQRDIITGWKKFVDRKKTSGIC (*Arabidopsis* GAD1)	5	P, No CaMBD	Baum et al. (1993), Baum et al. (1996)
Apyrase	FNKCKNTIRKALKLNY (Pea)	2	P	Hsieh et al. (2000), Steinebrunner et al. (2003)
Catalase	GIRTKCVIKKEEENNFKQAGDRYRSWAPDRQDRFVKRWV (*Arabidopsis* Cat3)	2	P, No CaMBD	Yang and Poovaiah (2002b)
Auxin-induced protein ZmSAUR1	NKIRDIVRLQQLLKKWKKLATVTPSA (Maize)	>30	NP	Yang and Poovaiah (2000a)
DNA-binding protein CGCG	IWSVGILEKVILRWRRKGSGLRGFK (*Arabidopsis* AtSR1)	6	P	Yang and Poovaiah (2002a), Du et al. (2009)
Nuclear protein PCBP	SKLKKLILLKRSIKALEKARKF (Potato)	3	NP	Reddy et al. (2002)
Disease resistance gene MLO	MKALMNWRKKAMEKKKVR (Rice OSMlo)	15	P	Kim et al. (2002b), Kim et al. (2002a)
Cyclic nucleotide gated cation channels (CNCG)	FRRLHSKQLRHTFRFYSGQWRTW (Tobacco NtCBP4)	20	P	Arazi et al. (1999), Kohler et al. (1999), Ali et al. (2007)
Vacuolar Ca^{2+}-ATPase	RQRWRSSVSIVKNRARRFRMISNL (Brassica)		NP	Malmstrom et al. (2000)
ER Ca^{2+}-ATPase	LEKWRNLCGVVKNPKRRFRFTANL (*Arabidopsis* AtACA2)		NP	Hong et al. (1999)
Plasma membrane Ca^{2+}-ATPase[b]	VLQRWRRLCGIVKNPRRRFRF (SoybeanSCA1) RRTIQFKLRIAILVSKA (Soybean SCA1)	14	P	Chung et al. (2000)
AAA family CIP111	LWTPLKSVAMFLRRHIAS (*Arabidopsis*)	1	P, No CaMBD	Zielinski (2002)
AAA+−ATPase AFG1L1	RSRWFWSRLMPQTSYSPV	1	P, No CaMBD	Bussemer et al. (2009)
Kinesin-like protein	ISSKEMVRLKKLVAYWKEQAGKK (*Arabidopsis*)	1	NP	Reddy et al. (1996), Wang et al. (1996)

Chimeric Ca^{2+}/CaM-dependent protein kinase (CCaMK/DMI3)	SFNARRKLRAAAIASVLSS (lily) (*Medicago truncatula*)	Not in Brassica family	NP	Patil et al. (1995), Takezawa et al. (1996), Gleason et al. (2006), Tirichine et al. (2006)
Diacylglycerol kinase (LeCBDGK)	KRQNRSHGRKPRLWALLRNLLAFRLERH (Tomato)	3	P, No CaMBD	Snedden and Blumwald (2000)
Pollen-specific protein (MPCBP, NPG1)	VSKGWRLLALILSAQQRF (Maize) VLKGWRFLALVLSAQQRF (*Arabidopsis*)	3	NP	Safadi et al. (2000)
Heat shock repressed protein TCP60	GWLKIKAAMRWGFFVRKKA (Tobacco)	6	NP	Lu and Harrington (1994)
CBP60g	RNLTFKKVVKKVMRDQSNNQFMIQ (*Arabidopsis*)	6	NP	Wang et al. (2009)
Chaperonin 10	LYSKYAGNDFKGKDGSNYIALRASDVMAILS (*Arabidopsis*)	1	NP	Yang and Poovaiah (2000c)
AtBT	KMVEDTKWKVLVRRVASAKAMSSL (*Arabidopsis*, AtBT1)	5	NP	Du and Poovaiah (2004)
Cytoplasmic Receptor-like kinase (CRCK1)	FKGKLDDGTIVAIKRARKNNYGKS		No	(Yang et al. 2004)
NAD kinase (NAD2)	IYVHSKEGVWRTSAMVSRWK	2	P	Turner et al. (2004)
FAD-dependent oxidoreductase, DWF1	CRKKYRAIGTFMSVYYKS	1	P, No CaMBD	Du and Poovaiah (2005)
WRKY group IId AtWRKY7	VAVNSFKKVISLLGRSR	8	No	Park et al. (2005)
NADPH-dependent dehydrogenase Tic32	DVDLAKKLWDFSINLVK	1	No	Chigri et al. (2006)
Ubiquitin-specific protease 6 (AtUBP6)	SQFWMVLRKKYPQFSQLQNG	1	P	Moon et al. (2005))
NPK phosphatase (NtMKP1)	NGWSRLRRFKSSGIMKEFITASK	1	No	Yamakawa et al. (2004), Ishida et al. (2009)
Receptor-like kinase (CRLK1)	MRDIVQVLTRVIKVRHCRKRQK (C-terminal) FRYHRKKSQIVNSGSRRSAT (N-terminal)	2	No	Yang et al. (2010)

[a]P: present in nonplant systems; NP: not present in nonplant systems; P, No CaMBD: present but no calmodulin-binding domain in nonplant systems
[b]Soybean SCA1 has two calmodulin-binding domains

peptides (Hoeflich and Ikura 2002; Yang and Poovaiah 2003; Bouche et al. 2005; Yamniuk et al. 2007). Relieving autoinhibition, a characteristic "wrap-around" binding mode, is the classical mechanism for the calmodulin-dependent regulation of target proteins in animals (Kurokawa et al. 2001; Hoeflich and Ikura 2002; Ikura and Ames 2006). Recently, a relieve autoinhibition by calcium/calmodulin binding to plant glutamate decarboxylase (GAD) has been observed. GAD is an approximately 340-kDa hexamer, and its activity is regulated by the binding of calcium–calmodulin to the C-terminal domain where a basic amphipathic alpha helix structure is projected (Fig. 2a). The N- and C-lobes of calcium–calmodulin can each simultaneously bind to identical peptides corresponding to the calmodulin-binding site to form a 1:2 calcium–calmodulin:GAD complex (Fig. 2b), which suggests that calcium–calmodulin promotes the GAD dimerization (Yap et al. 2003). However, after analyzing the low-resolution structure of the calmodulin-activated GAD complex using small-angle X-ray scattering, the crucial residues in the C-terminal calmodulin-binding site for regulation by calmodulin as well as pH have been found (Gut et al. 2009). Calmodulin activates hexamer GAD in a unique way by relieving two C-terminal autoinhibition domains of adjacent active sites, forming a 393-kDa GAD-calmodulin complex with an unusual 1:3 stoichiometry (Fig. 2c). The complex is loosely packed due to the flexible links connecting the enzyme core with the six C-terminal regulatory domains of GAD (Fig. 2d). This represents a complicated relief autoinhibition mode. In a simple case, calcium/calmodulin relieves autoinhibition by binding to a short (20–25 residues) calmodulin-binding domain (CaMBD) sequence that is adjacent to or within an autoinhibitory region of the enzyme (Fig. 3). Typically the calmodulin C-lobe binds with high affinity to a hydrophobic residue such as Trp residue within the target sequence, and the flexible central link allows the N-lobe to bind to a second bulky hydrophobic "anchor" residue within the target sequence.

Recent studies on calmodulin binding to target proteins/peptides including plant-specific targets indicate that there are more calmodulin regulatory mechanisms. Calcium/calmodulin can compete with the ligand binding site in the potato kinesin-like CaM-binding protein (KCBP) (Vinogradova et al. 2004) and plant cyclic-nucleotide gated channels (CNGCs) (Arazi et al. 2000). Since the Ca^{2+}/CaM-binding site on the target protein overlaps with the respective ligand binding site, calcium/calmodulin binding to KCBP and CNGC could prevent their binding from microtubules and cyclic nucleotide monophosphates, respectively (Fig. 3). In the case of the active site remodeling for the activation of anthrax adenyl cyclase, the N- and C-lobes of calmodulin can bind to two distinct regions of the adenylyl cyclase enzyme and induce a conformational reorganization that creates the enzyme's active site (Drum et al. 2002). This has been described as a phenomenon of "man bites dog" (Meador and Quiocho 2002). Interestingly, in this study calcium did not bind to all four EF hands of calmodulin. Only the C-lobe of calmodulin is in a canonical Ca^{2+}-bound open conformation, whereas the N-lobe remained Ca^{2+} free with a closed conformation (Fig. 3). Calcium–calmodulin also can induce the target dimerization, as appearing in small conductance Ca^{2+}-activated K^+ channels to regulate the channel activity. Two calmodulins and two channel proteins form a 2:2 complex

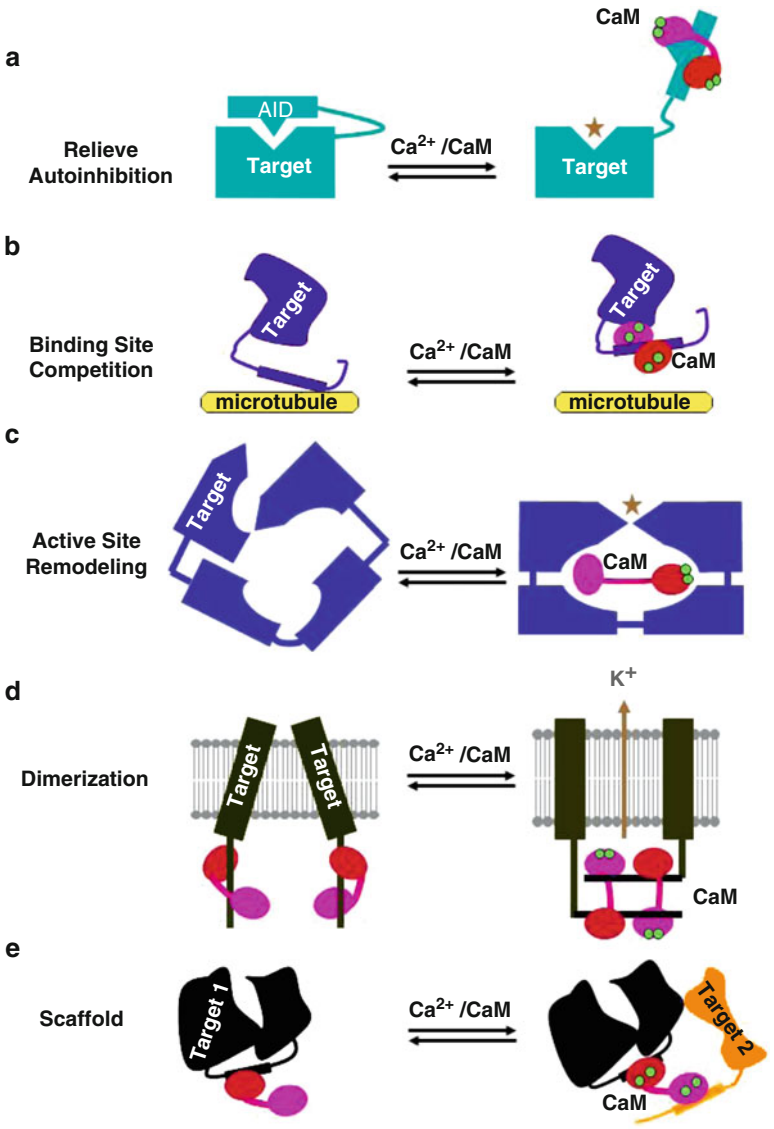

Fig. 3 Schematic model for the various mechanisms of calmodulin-dependent target regulation. (**a**) Relieving autoinhibition: calcium/calmodulin binding to the target induces a conformational change to displace the autoinhibitory domain (AID) and allows for full activity. (**b**) Binding site competition: calcium/calmodulin binding to a site overlapping with other ligand binding site such as in the case of the microtubule-binding face in a potato kinesin. (**c**) Active-site reorganization: upon calcium/calmodulin binding, a helical domain of oedema factor undergoes a rotation away from the catalytic core, which stabilizes a disordered loop and leads to enzyme activation. (**d**) CaM-induced target protein dimerization: two calmodulins interact with two potassium channel domains upon calcium binding. Two calcium ions only bind to N-terminal EF hands

(Schumacher et al. 2001), where calcium is bound to the N-lobe EF hands, but not to the C-lobe EF hands of calmodulin (Fig. 3). The N-terminal EF hands are responsible for calcium-induced dimerization leading to channel gating and direct coupling between changes in intracellular calcium concentrations and altered membrane potential.

Further a sequential target binding mode for calcium/calmodulin binding to the calmodulin-binding domain region of NtMKP1, a *Nicotiana tabacum* MAPK phosphatase-1, was observed (Ishida et al. 2009) where calmodulin could act as a scaffold protein to recruit two different calmodulin-binding proteins (Fig. 3). The 12-residue calmodulin-binding domain in NtMKP1 was found to bind exclusively to the C-lobe of SCaM4. Specific Trp and Leu side chains facilitate strong binding through a "double anchor" motif. The orientation of the helical peptide on the surface of calcium-SCaM4 is distinct from other known complexes. The N-lobe of calcium-SCaM4 in the complex remains free for recruiting another target protein. Since calmodulin does not stimulate the phosphatase activity, it is suggested that calmodulin could possibly act as a calcium-dependent adaptor protein. However, the second target protein remains to be identified. There are examples of related EF-hand proteins acting as adaptor proteins, including centrin and calcium- and integrin-binding protein 1 (Sheehan et al. 2006; Tsuboi et al. 2006).

In addition, plants have many proteins carrying IQ motif(s). IQ motif was first discovered in the calcium-independent calmodulin-binding proteins such as myosins in animals (Xie et al. 1994). It carries a common feature of IQXXX-RGXXXR where X is any amino acid. In plants, many proteins with IQ motifs have been detected, and some of them are calmodulin-binding proteins. Calmodulin binding to IQ proteins can be either calcium independent such as AtBAG6 (Kang et al. 2006) or calcium dependent such as IQD1 (Levy et al. 2005). Therefore, considering there are multiple calmodulin isoforms and a large number of calmodulin-binding protein populations, it is reasonable to believe that more calmodulin modulation modes are likely to be discovered.

2 Calmodulin, Calmodulin Isoforms, and Calmodulin-Like Proteins

The Ca^{2+}/CaM-signaling systems in plants and animals share considerable similarity in fundamental aspects, but a much more complicated system is being elucidated in plants. Usually there are three different genes coding for one identical calmodulin protein in vertebrates; however, there are multiple calmodulin isoforms and

Fig. 3 (continued) which are responsible for calcium-induced dimerization leading to channel gating. (**e**) Calmodulin acting as a scaffold to bring two calmodulin-binding proteins together. Parts (**a**), (**c**), and (**d**) are adapted from Yang and Poovaiah (2003), and (**b**) and (**e**) are adapted from Yamniuk et al. (2007)

calmodulin-like proteins (CMLs) in plants. In *Arabidopsis*, there are seven calmodulin genes coding for four isoforms which share very high sequence similarity with calmodulin from vertebrates. AtCaM7 shares the highest similarity (89.3%, 133 out of 149 aa identical) to human calmodulin; AtCaM6 codes for a different isoform, AtCaM1 and 4 for the third CaM isoform, and AtCaM2, 3, and 5 for the fourth calmodulin isoform. All these calmodulin isoforms are 149 aa long and the last three calmodulin isoforms share 132 aa identity with human CaM. Most of the sequence variations between these *Arabidopsis* calmodulin isoforms and vertebrate calmodulins are conservative, and therefore these proteins are considered to be true CaMs and should share similar function to that of their animal counterparts (McCormack and Braam 2003; McCormack et al. 2005).

In addition, there are about 50 CaM-like proteins in *Arabidopsis* which share similar sequence and structural features with calmodulin in that they are composed of varying number of EF hands, contain no other known or identifiable functional domains, and share at least 16% amino acid identity with vertebrate CaMs. CML1 is the only member carrying a single EF hand, and the rest of CMLs have at least two identifiable EF hand-like motifs. Most CMLs carry 4 EF hands, and CML12, also known as TCH3, is the only one which has six EF hands (McCormack et al. 2005). Furthermore, there are even species-specific CaM-like proteins which exist in some species and absent from others. For example, petunia PhCaM53 and rice OSCaM61 are special calmodulin-like proteins which resemble each other and carry a stretch of basic amino acid extension at the C terminus. An ortholog of PhCaM53 or OsCaM61 does not exist in *Arabidopsis* (Rodriguez-Concepcion et al. 1999; Dong et al. 2002). rgs-CaM from tobacco is another example of a species-specific CaM-related protein with three EF hands and a 50-amino acid long N-terminal extension. rgs-CaM was found to act as a *r*egulator of *g*ene *s*ilencing in tobacco, and its homolog is absent in *Arabidopsis* genome (Anandalakshmi et al. 2000). Cultivated hexaploid wheat has one of the most complicated genomes and carries the largest known family of CaM- and calmodulin-like genes. TaCaM-III, a CaM-like protein, has a unique structural feature in that it lacks the first EF hand and contains a hydrophobic domain with a tryptophan residue instead (Yang et al. 1996).

The existence of multiple calmodulin isoforms and large families of calmodulin-like proteins including species-specific isoforms is the most obvious and yet fascinating difference of Ca^{2+}/CaM-mediated signaling in plants as compared to animals. It is generally accepted that structural variations in proteins are the basis for their functional differences. Structural changes such as variations in N- or C-terminal extensions, variations in the hinge joint of the N- and C- lobe, and variations in joints between EF hands of CaMs and CMLs all carry functional impacts in terms of differences in calcium-binding affinity, selection and activation of target proteins, etc. (Reddy 2001a; Yang and Poovaiah 2003; Yamniuk and Vogel 2004; Bouche et al. 2005; McCormack et al. 2005). It has recently been reported that even a single amino acid difference between CaM7 and CaM2/3/5 in the fourth EF hand could make a difference in their ability to interact with a particular target, the Z-/G-box DNA motif (Kushwaha et al. 2008). It is well

documented that distinct plant CaM isoforms differ in their ability to bind and activate known CaM-regulated enzymes *in vitro* (Reddy et al. 1999; Lee et al. 2000; Choi et al. 2002). The differential CaM/CML and target interactions and regulations have also been observed under *in vivo* conditions during the regulation of AtNHX, a vacuolar Na^+/H^+ exchanger from *Arabidopsis* (Yamaguchi et al. 2005). In addition, various CaMs and CaM-like proteins might also have differential affinities to Ca^{2+}, due to the variation in the number of EF-hand motifs, or mutations in the sequence of EF-hand motifs, the most conservative portion in calmodulins and calmodulin-like proteins. Although most of the EF-hand motifs in calmodulin-like proteins retain the canonical Ca^{2+}-binding coordination comprising of a loop structure with 12 reserved residues between the E and F helices, several CMLs carry a loop structure slightly different from those of typical calmodulins with an E to D substitution in position 12 of the Ca^{2+} binding loop (McCormack and Braam 2003). Research on the interaction of EF hands in parvalbumin and divalent cations revealed that E to D substitution in this position might lower its binding affinity to Ca^{2+} and increase its affinity to Mg^{2+} (Cates et al. 2002). Hence the E to D substitution in these CML might affect its interaction with Ca^{2+} and render the ability for these CMLs to bind to other divalent cations such as Mg^{2+} although it remains to be confirmed. Furthermore, variation in CaMs/CMLs could even be correlated with their subcellular localization. Generally, CaM is a cytosolic protein, but CaM and CML have also been found in the nucleus (van der Luit et al. 1999), the peroxisome (Yang and Poovaiah 2002b), vacuole (Yamaguchi et al. 2005), and even in the extracellular matrix (Ma et al. 1999). The necessity of multiple subcellular locales is understandable because the CaM-target proteins are present in different subcellular locations. However, the manner in which CaM targets these organelles is not clear. In at least one case, posttranslational modification correlated with structural variations in CaM or CMLs could serve to control its subcellular localization. CaM53 from petunia (PhCaM3) is a calmodulin-like protein with an extra extension in its C-terminal region which is rich in basic amino acid and also carries a CTIL CaaX-box motif. It was shown to associate with the plasma membrane when prenylated and target to the nucleus if prenylation was inhibited (Rodriguez-Concepcion et al. 1999).

Why multiple CaM and CML genes encode for the same or similar proteins in plants are not fully understood. Although the functional redundancy between different members cannot be excluded, accumulated evidence suggests that each CaM genes could have unique functional application and significance, revealing a much more complicated signaling system required to support a sessile life style in plants in which environmental changes bring forth endless challenges. This is further supported by the differential expression patterns of CaMs/CMLs. In all plants examined, CaM genes, even genes encoding the same isoform, are differentially expressed in response to numerous external stimuli such as touch, heat shock, cold, light, and pathogens, and to hormones such as auxin (Reddy 2001a; Yang and Poovaiah 2003; Bouche et al. 2005). CaMs are also differentially expressed in different stages, as well as in different tissue and cell types (Yang et al. 1998; Duval et al. 2002; Yang and Poovaiah 2003). The complexities in the interpretation

of Ca^{2+}-mediated signals through multiple calmodulin isoforms and large calmodulin-like protein family are clearly demonstrated in the recent progresses in functional characterization of proteins interacting with/regulated by CaMs and CMLs.

3 Functional Implication of CaM and Target Interactions

Since CaM does not contain known functional structural feature, CaM itself usually has no particular enzymatic function. However, when loaded with Ca^{2+}, CaM can bind to and regulate the function of its targets through conformational changes (Chin and Means 2000; Hoeflich and Ikura 2002; Yang and Poovaiah 2003; Bouche et al. 2005). Hence, the functional significance of calmodulin is usually hosted by its various targets, making calmodulin an omnipotent signaling component. Accumulated data have revealed that CaMs and CMLs are broadly involved in various cellular activities related to almost all kinds of physiological activities in plants' lives.

3.1 Ca^{2+}/Calmodulin-Dependent Regulation of Protein Phosphorylation in Plants

After the initial discovery of Ca^{2+}-stimulated phosphorylation in plants (Veluthambi and Poovaiah 1984), there has been an explosion of literature on Ca^{2+}-regulated protein phosphorylation in plants (for reviews see Poovaiah and Reddy 1987; Sathyanarayanan and Poovaiah 2004; Luan 2009; DeFalco et al. 2010). The cloning and characterization of the first plant calmodulin (Jena et al. 1989) and the first calmodulin (CaM) regulated protein kinase- chimeric calcium calmodulin dependent protein kinase (CCaMK) from lily (Patil et al. 1995) are two major advances in the study of Ca^{2+}/CaM regulated phosphorylation in plants. These discoveries suggested Ca^{2+}/CaM and Ca^{2+}/CaM regulated kinases that are critical molecular regulators of decoding Ca^{2+} signals into phosphorylation signals in animals are also present in plants. These findings enlightened our understanding of the evolution of Ca^{2+} and Ca^{2+}/CaM-regulated phosphorylation in plants and animals. Plants not only possess all the major components of Ca^{2+} and Ca^{2+}/CaM-dependent decoding of signals into phosphorylation signals but also demonstrate several structural specializations such as association of novel regulatory domains, as in the case of CCaMK, and groups of kinases that are directly regulated by Ca^{2+} binding (Ca^{2+}-dependent protein kinases, CDPKs).

In general, Ca^{2+}/CaM-dependent protein kinases (CaM kinases) have three functional domains: a serine-threonine kinase domain, an autoinhibitory domain, and a CaM-binding domain as found in CaMK I and CaMK IV from animal systems and CaMK from apple (Watillon et al. 1993). CaMK II from animals has an

additional domain at the C-terminal end known as association domain, which is involved in oligomerization of kinase (Colbran et al. 1989; Hanson and Schulman 1992). CaMK II holoenzyme consists of homooligomers or heterooligomers of 6–12 subunits each (Colbran et al. 1989; Hanson and Schulman 1992). Unlike other CaM-dependent kinases described above, CCaMK from plants has an additional domain known as a "visinin-like domain" at the C-terminal end. This domain is highly similar to visinin-like proteins from animal systems and has three EF hands and regulates kinase activity by regulating Ca^{2+}-stimulated autophosphorylation (Patil et al. 1995; Takezawa et al. 1996; Ramachandiran et al. 1997; Sathyanarayanan et al. 2000). A model for the regulation of kinase activity of CCaMK by Ca^{2+} , Ca^{2+}/CaM, and visinin-like domain is shown in Fig. 4.

Unlike CCaMKs, CDPKs do not contain a calmodulin-binding domain. CDPKs have three functional domains: a serine-threonine kinase domain, a junction domain, and a CaM-like domain with four EF hand Ca^{2+}-binding sites (Harper et al. 1991; Roberts and Harmon 1992; Harmon et al. 2000). The Ca^{2+}-binding domain of the archetypal CDPK has about 40% identity with CaM. Intriguingly,

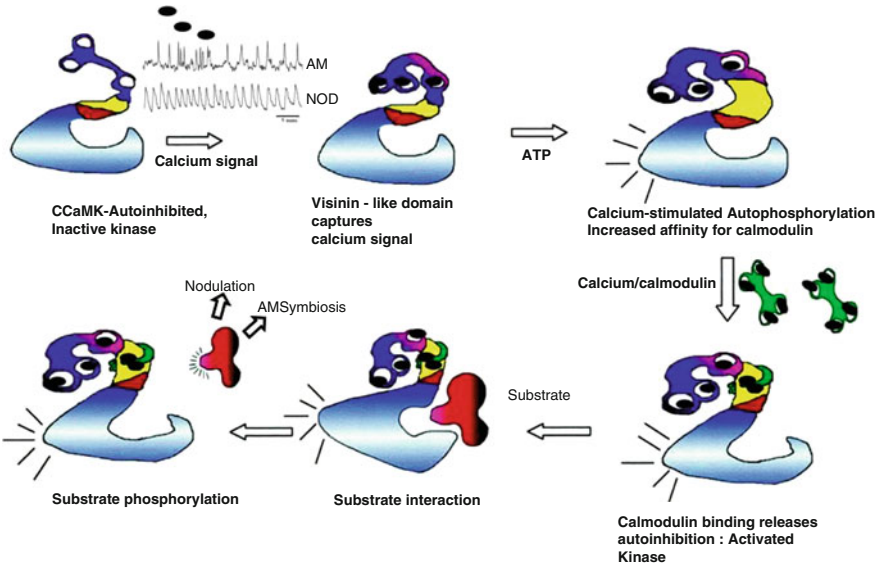

Fig. 4 Schematic illustration of CCaMK-mediated signaling and activation. In the rest condition, activities of CCaMK are suppressed by an autoinhibitory mechanism. Ca^{2+} transients induced by symbiotic microorganisms are perceived by both CaM and CaMK. The binding of Ca^{2+} to the visinin-like domain results in the autophosphorylation of CCaMK which drastically increases its affinity to Ca^{2+}/CaM. Binding of Ca^{2+}/CaM to CCaMK finally displaces the pseudosubstrate domain (autoinhibition domain) and results in the phosphorylation of different substrates based on the nature of the activating Ca^{2+} signatures. The phosphorylation of different substrates will determine whether a nodulation or mycorrhizalation pathway is activated. *Yellow* represents the CaM-binding domain, and *red* represents the autoinhibitory domain. *White circles* represent Ca^{2+}-binding EF hands in CCaMK and calmodulin

CDPK-like sequences are not present in the completed genome sequences of yeast (*Saccharomyces cerevesiae*), nematode (*Caenorabditis elegans*), fly (*Drosophila melanogaster*), and human (*Homo sapiens*). Another group of protein kinases similar to CDPKs is also present in plants. These CDPK-related protein kinases (CRKs) have a catalytic domain closely related to those of CDPKs, and their C-terminal domain has 20% identity with CaM (Lindzen and Choi 1995; Furumoto et al. 1996; Harmon et al. 2000).

After the first discovery of a CaM-regulated kinase in Apple and lily, several such kinases were discovered in recent years and their functions analyzed. Below we discuss our latest understanding of Ca^{2+}/CaM-regulated protein phosphorylation and its function in plants.

3.1.1 Ca^{2+}/CaM-Dependent Kinases in Regulating Growth and Development

In situ and immunohybridization experiments have shown that Ca^{2+}/CaM-dependent kinases are expressed in reproductive tissues and meristematic regions. For example, tobacco CCaMK is expressed in tapetal cells and its expression is regulated during microsporogenesis (Poovaiah et al. 1999). Another tobacco Ca^{2+}/CaM-dependent kinase from tobacco (NtCBK1) mRNAs is accumulated in the shoot apical meristem during vegetative growth but the levels are decreased after floral determination (Hua et al. 2004). This kinase, unlike CCaMK, does not contain a visinin-like domain. Interestingly, overexpression of NtCBK1 delayed transition to flowering suggesting a major role for this Ca^{2+}/CaM-dependent kinase as a negative regulator of flowering.

Ca^{2+}/CaM-dependent kinase from rice (OsCBK) is also expressed in reproductive and vegetative tissues of rice and showed temporal and spatial changes during growth and development (Zhang et al. 2002). OsCBK is highly expressed in zones of cell division.

Two other major functions of Ca^{2+}/CaM-dependent kinases are mediating self-incompatibility and symbiosis. *Brassica oleracea* S locus receptor kinase has been shown to interact with CaM (subdomain VIa) and this binding site is conserved in the plant receptor kinase family (Vanoosthuyse et al. 2003). CBRLK1 (*C*almodulin-*b*inding *r*eceptor-*l*ike protein *k*inase) is a membrane-bound CaM-kinase and was classified into S-locus RLK family (Kim et al. 2009a). A CaM-binding domain is present at the C-terminus of CBRLK1. AtCaMRLK (*Arabidopsis* calmodulin-binding RLK) is expressed in all reproductive and vegetative tissues except leaves (Charpenteau et al. 2004) and contains a single CaM-binding region.

Nod factor signaling in leguminous plants leads to the induction of Ca^{2+} oscillations (Ehrhardt et al. 1996) and this transduction requires DIM3 (Levy et al. 2004; Mitra et al. 2004). DIM3 encodes a Ca^{2+}/CaM-dependent protein kinase. Removal of the autoinhibition domain resulted in autoactivation of nodulation signaling (Gleason et al. 2006) suggesting that Ca^{2+}/CaM binding to kinase that releases autoinhibition (Takezawa et al. 1996; Sathyanarayanan et al. 2000) is a central switch for nodule morphogenesis. The importance of CCaMK-mediated

processes in nodule morphogenesis is further illustrated by a study by Tirichine et al. (2006) who show that a single amino acid replacement in CCaMK turned on fully differentiated cortical cells into meristematic founder cells of nodule primordia and resulted in spontaneous nodule formation (Tirichine et al. 2006).

3.1.2 Ca^{2+}/CaM-Dependent Kinases in Regulating Stress Responses

Ca^{2+}/CaM-dependent kinases have been shown to play a crucial role in mediating several stress responses such as cold, temperature, and salinity. Ca^{2+}/CaM-regulated receptor-like kinase CRLK1 is present on the plasma membrane (Yang et al. 2004, 2010) and is crucial for cold tolerance in plants. Unlike other Ca^{2+}/CaM-dependent kinases, CRLK1 has two CaM-binding sites each with a different affinity for Ca^{2+}/CaM (25 nM and 160 nM). CRLK1 knockout mutants exhibited an increased sensitivity to chilling and freezing temperatures. Cold response genes such as CBF1, RD29A, and COR15a showed a delayed response in CRLK1 knockout plants suggesting a critical role for CRLK1 in regulating cold tolerance. The pea CCaMK was upregulated in roots in response to low temperature and increased salinity (Pandey et al. 2002).

Calmodulin-binding protein kinase 3 (AtCBK3) in *Arabidopsis* interacts with heat-shock transcription factors (Liu et al. 2008). T-DNA insertion knockouts of AtCBK3 result in impaired basal thermal tolerance which could be complemented by insertion of wild-type AtCBK3 into mutant plants.

3.1.3 Ca^{2+}/CaM-Dependent Kinases: Mechanisms of Specificity

Decoding of Ca^{2+} Signals: Ca^{2+}, Ca^{2+}/CaM binding, and autophosphorylation are described as the three steps critical to activating CCaMK (Sathyanarayanan et al. 2000, 2001). These seemingly simple steps are in fact complicated by the possibility of multiple autophosphoryaltion sites, differential sensitivity to Ca^{2+} and to Ca^{2+}/CaM.

Autophosphorylation: An important consequence of Ca^{2+} or Ca^{2+}/CaM activation of protein kinases is the autophosphorylation of kinases. In general, autophosphorylation functions as a regulatory switch to activate the kinase, enabling the kinase to phosphorylate its substrates. Thus it is important to identify specific sites for autophosphorylation. Trypsin digestion of autophosphorylated kinase followed by mass spectrometry was used as a major approach to identify critical residues of autophosphorylation of lily CCaMK (Sathyanarayanan et al. 2001). This strategy has been successful in large-scale identification of critical residues in several CDPKs (Hegeman et al. 2006). Interestingly the Ca^{2+}-dependent autophosphorylation of lily CCaMK resulted in time-dependent loss of activity suggesting an important mechanism regulating activity of CCaMK (Sathyanarayanan and Poovaiah 2002).

Differential Ca^{2+} *sensitivity:* As we previously described in an earlier review (Sathyanarayanan and Poovaiah 2004), calcium signals may be briefly modulated in their amplitudes or their frequency, a potential mechanism of imparting specificity to signaling in differential Ca^{2+} sensitivity. We found that this is true in the case of the three EF hands at the C-terminal visinin-like domain of lily CCaMK. The three EF hands of CCaMK have differential binding affinity (from nanomolar to micromolar) for Ca^{2+} (unpublished observation). This is also true in the case of different CDPKs (Lee et al. 1998). CDPK alpha, beta, and gamma isoforms showed 51, 1.4, and 1.6 μM affinities for Ca^{2+}.

Differential CaM-binding affinity: A given cell could express different CaM isoforms that function as major receptors for Ca^{2+}. Differential affinities for CaM isoforms help the kinase to transduce specific calcium transients. For example potato calmodulin 1 and 6 showed differential effects in regulating Ca^{2+}/CaM-dependent substrate phosphorylation of tobacco CCaMK (Liu et al. 1998).

Tobacco calmodulin-dependent kinase 2 (NtCBK2) also displayed differential binding affinities for the three tobacco CaMs (Hua et al. 2003b). The dissociation constants are NtCaM1: 55.7 nM, NtCaM3: 25.4 nM, and NtCaM 13: 19.9 nM.

One kinase-one signal: Another mechanism of conferring specificity is differential activation of kinases by different signals. For example despite more than 91% overall sequence identity, tobacco calcium-dependent protein kinases (NtCDPK1 and NtCDPK2) are differentially phosphorylated in vivo in response to biotic or abiotic stress. In NtCDPK2, serine 40 and threonine 65 were phosphorylated within 2 min after stress (Witte et al. 2010) whereas serine 54 (a site that is absent in NtCDPK2) was phosphorylated in NtCDPK1.

3.1.4 Regulation of Phosphorylation by Ca^{2+} and Ca^{2+}/CaM-Dependent Protein Phosphatases

Ca^{2+} signals can also be decoded by specific dephosphorylation of phosphoproteins. Calcineurin B-like proteins (CBL) are unique Ca^{2+} sensors in plants (Kudla et al. 1999; Luan et al. 2002). The CBL proteins regulate a unique family of protein kinases (CBL-interacting protein kinases or CIPKs). *Arabidopsis* has multigene families of CBL and CIPKs (Cheong et al. 2003; Kim et al. 2003; Pandey et al. 2004). CBL1 and CBL9 function in responses to ABA (Cheong et al. 2003; Pandey et al. 2004). Li et al (2006) reported CIPK23, which interacts with CBL1 and CBL9, to be critical for plant growth in low K media and for stomatal regulation (Li et al. 2006). Furthermore, CIPK23 phosphorylates a voltage-gated inward K channel required for K^+ acquisition in *Arabidopsis*. Interestingly, when CBL5 was overexpressed in *Arabidopsis* (Cheong et al. 2010), plants showed enhanced tolerance to high salt or drought stress. Overexpression of CBL5 also leads to gene expression of stress markers RD29A and RD29B.

A PP2C-like phosphatase with a CaM-binding domain (PCaMPP) was isolated from moss *Physcomitrella patens* by using radiolabeled CaM (Takezawa 2003). PCaMPP has a catalytic domain similar to serine threonine phosphatase and has a

functional CaM-binding domain at the C-terminus. Homologs of PCaMPP are present in the genomes of higher plants but their physiological functions are yet to be determined. A dual specificity protein phosphatase with two CaM-binding domains in *Arabidopsis* was also identified by screening of an expression library (Yoo et al. 2004).

3.2 Ca^{2+}/CaM-Mediated Transcriptional Control

Transcriptional control is one of the major approaches for calmodulin to regulate cellular activities in response to various environmental and developmental cues. This notion has been demonstrated by the increasing list of calmodulin-binding proteins which are involved in transcriptional controls, such as kinases, transcription factors, transcription cofactors as well as nuclear proteins with unknown biochemical functions including PCBP, AtCaMBP25, etc. The unfolding picture revealed that calmodulin could regulate transcription through multiple approaches.

3.2.1 Regulating Transcription Through Kinase Cascades

CaM can regulate transcription activators indirectly through CaM kinases and the phosphatase calcineurin in animals (Hoeflich and Ikura 2002), and similar regulation has recently been observed in plants. AtCBK3 (also called AtCRK1, CDPK related kinase 1) contains a serine-threonine kinase domain in the N-terminus, a CaM domain binding domain in the middle followed by a C-terminal extension with degenerate EF hands which likely lose their normal function. AtCBK3 binds CaM in a Ca^{2+}-dependent manner. Biochemical analysis showed that the kinase activities of AtCBK3 in terms of autophosphorylation and substrate phosphorylation of histone IIIS and syntide-2 were not affected by Ca^{2+} alone, but were stimulated by several Ca^{2+}-loaded CaM isoforms to similar extents (Wang et al. 2004). Very recently, AtCBK3 was found to interact with heat-shock transcription factor AtHSFA1a (also called AtHSF1). Phosphorylation assay showed that recombinant AtCBK3 phosphorylates recombinant AtHSFA1a in vitro in a Ca^{2+}/CaM-stimulated manner, indicating AtHSFA1a is a substrate of AtCBK3. The knockout mutant of AtCBK3 had impaired thermotolerance, which could be rescued by complementation with AtCBK3 cDNA. Overexpression of AtCBK3 resulted in elevated thermotolerance in the transgenic plant. Furthermore the modified thermotolerance in AtCBK3 knockout mutant and overexpression lines were found to be correlated with the decreased and increased binding of heat-shock element (HSE) to the total protein preparations from the compared lines as well as the down- and upregulated expression of HSPs including AtHSP18.2, AtHSP25.3, AtDjA2, and AtHSP83 in these plants. These results suggested that Ca^{2+}/CaM could regulate the activities of protein kinase AtCBK3 which phosphorylates transcription factor AtHSFA1a to increase its interaction with HSE and stimulate the expression of HSPs (Liu et al. 2008).

PP7 is a protein Ser/Thr phosphatase which interacts with CaM in a Ca^{2+}-dependent manner (Kutuzov et al. 2001). The expression of the AtPP7 gene was also induced by heat shock (HS) at 37°C in wild-type *Arabidopsis*. A recent functional characterization of AtPP7 revealed that AtPP7 knockout mutant is impaired in its thermotolerance while the overexpression of AtPP7 resulted in increased thermotolerance, and expression of AtHSP70 and AtHSP101 genes was upregulated in these AtPP7 overexpression lines upon heat-shock treatment. Interestingly, AtPP7 was also found to interact with AtHSF1 (also called AtHSFA1a), implying that AtPP7 could also regulate AtHSF1 (Liu et al. 2007), although detail about how AtPP7 dephosphorylated AtHSF1 and regulates its function is basically unaddressed.

3.2.2 Regulating Transcription Through CaM-Binding Transcription Cofactors

Using protein–protein interaction-based screening of an *Arabidopsis* cDNA expression library, five AtBT genes were found to code calmodulin interaction proteins with conserved structure feature: a BTB domain in the N-terminus and a Zf-TAZ domain in the C-terminus. The AtBT1 was found to target the nucleus and interact with bromodomain transcription regulator AtBET10, indicating a role for BTs in transcription regulation. The interaction between AtBT1 and AtBET10 is likely conserved between AtBT and AtBET members (Du and Poovaiah 2004). While searching for downstream targets of transcription factor telomerase activator1 (TAC1), BT2 was found to be a downstream target of TAC1 critical for the induced expression of AtTERT, the catalytic unit of telomerase complex (Ren et al. 2007). TAC1-mediated activation of telomerase positively correlates with auxin treatment, a process reported to elevate intracellular Ca^{2+} transients (Shishova and Lindberg 2004). Interestingly, treatment with calcium ionophore enables TAC1 to activate telomerase in lower auxin condition, and like *TAC1*, enhanced expression of *BT2* was also found to exacerbate the high-auxin phenotype of the *yucca* mutant (Ren et al. 2007). BT2 was also found to suppress sugar- and ABA-mediated responses during germination (Mandadi et al. 2009). In an independent study, BTs were found to target both nucleus and cytoplasm, and functional redundancy was observed among different BT members. Multiple loss-of-function mutations in BT genes revealed that BT proteins play a critical role during gametogenesis (Robert et al. 2008).

3.2.3 Calmodulin Binding and Regulation of DNA-Binding Transcription Factor

Arabidopsis TGA3, a basic leucine zipper transcription factor, is the first transcription factor reported to be regulated by calmodulin (Szymanski et al. 1996). TGA transcription factors including TGA3 are interaction partners of NPR1, a transcription

cofactor which carries an ankyrin-repeat and a BTB domain. Together, they play a critical role in controlling the expression of disease-resistance genes such as PR1, although the detailed mechanism deserves further elucidation (Durrant and Dong 2004). The CaM binding to TGA3 enhances its interaction with target promoter (Szymanski et al. 1996). Whether CaM binding to TGA3 has any impact on disease resistance remains unknown.

AtSRs/CaMTAs are the best characterized CaM-binding transcription factors in plants as well as in animals. The first report of an AtSR/CaMTA family member being a Ca^{2+}/CaM-binding protein was published in 2000 (Yang and Poovaiah 2000b). Accumulated data have revealed that AtSR/CaMTA homologs belong to a conserved family and exist in multicellular eukaryotes including plants, insects, and mammals (Reddy et al. 2000; Bouche et al. 2002; Yang and Poovaiah 2002a; Choi et al. 2005). AtSRs/CaMTAs share a conserved domain structure with a sequence-specific CG-1 DNA binding domain in the N-terminal region, followed by a transcription activation domain (TAD), a transcription factor immunoglobulin (TIG)-like nonspecific DNA-binding domain, ankyrin repeats (ANK), and tandem repeats of IQ motifs joined to a canonical calmodulin-binding domain (CaMBD) in the C-terminal region. During the last few years, there have been some exciting developments regarding the functional significance of this group of transcription factors. In *Arabidopsis*, two T-DNA knockout lines of AtSR1/CaMTA3 were shown to exhibit autonomous lesion and leaf chlorosis, elevated expression of pathogensis-related (PR) genes, and enhanced resistance against *Pseudomonas syringae*, a biotrophic pathogen (Galon et al. 2008; Du et al. 2009). The constitutive defense phenotypes displayed by AtSR1/CaMTA3 loss-of-function mutations were correlated with elevated accumulation of salicylic acid (SA), indicating that AtSR1/CaMTA3 acts as a negative regulator of SA-mediated immune responses (Du et al. 2009). Since SA is an adequate inducer of systemic acquired resistance (SAR) which is effective against a broad range of pathogens (Durrant and Dong 2004), it is not surprising to see that atsr1/camta3 null mutants also showed increased resistance to necrotrophic fungal pathogen *Botrytis cinerea* (Galon et al. 2008). A typical AtSR1/CaMTA3 recognition site exists in the −1 kb promoter region of EDS1, a critical player for the induced production of salicylic acid, and the transcription of EDS1 was found to be negatively regulated by AtSR1 in a calmodulin-binding-dependent manner (Du et al. 2009). OsCBT, an AtSR/CaMTA homolog in rice, was also found to act as a negative regulator of disease resistance in rice. A T-DNA insertion line, oscbt-1, exhibits a partial dwarf phenotype and enhanced resistance to both the rice blast fungus *Magnaporthe grisea* and bacterial pathogen *Xanthomonas oryzae* pv. oryzae (Koo et al. 2009). In a different line of study, the conserved DNA motif 2 (CM2: CCGCGT), a typical AtSR/CaMTA recognition sequence in the promoter of cold responsive CBF2, was found to confer both positive and negative regulation to the expression of CBF2 (Doherty et al. 2009). Expression of endogenous CBF2 is remarkably compromised in atsr1/camta3 null mutant, and this decrease can be restored by complementation of *AtSR1*/*CaMTA3* under the control of 35 S promoter or by introducing another null mutation in *AtSR2*/*CaMTA1*, implying that the transcription of *CBF2* is positively regulated by

AtSR1/*CaMTA3* and negatively regulated by *AtSR2*/*CaMTA1*. The negative regulation through CM2 motif by *AtSR2*/*CaMTA1* was also supported by the observation that the knockout of *AtSR2*/*CaMTA1* resulted in an obvious elevation of GUS expression driven by synthetic promoter containing a tetramer of a 27 core sequence covering CM2 from CBF2 promoter (Doherty et al. 2009). Although a single mutant of *AtSR1*/*CaMTA3* produced no difference in cold or freezing tolerance, the double mutant of *AtSR1*/*CaMTA3* and *AtSR2*/*CaMTA1* resulted in a compromise in freezing tolerance as compared with wild type, but the underlying mechanism deserves further attention (Doherty et al. 2009). Although in most cases AtSR/CaMTA homologs act as transcription activators, recent research on the regulation of innate target promoters showed that *AtSR1*/*CaMTA3* and *AtSR2*/*CaMTA1* could also act as transcription suppressors (Doherty et al. 2009; Du et al. 2009). In the tested cases, the functions of AtSRs/CaMTAs were found to be dependent on their interaction with Ca^{2+}/CaM (Choi et al. 2005; Han et al. 2006; Du et al. 2009). Based on accumulated knowledge, a model describing Ca^{2+}/CaM-mediated regulation of transcriptional control through AtSRs/CaMTAs in plants during plant responses to biotic and abiotic stresses is included in Fig. 5. Besides these significant roles of AtSRs/CaMTAs in plants, AtSRs/CaMTAs homologs also have critical roles in animals. Fruit fly dCaMTA was reported to stimulate the expression of dFbxl4 in controlling the deactivation of rhodopsin, a G protein-coupled light receptor (Han et al. 2006). Human CaMTA1 is expressed specifically in brain and was reported to be a potential suppressor of oligodendrogliomas (Barbashina et al. 2005) and neuroblastomas (Henrich et al. 2006). Mouse CaMTA2 has been reported to mediate cardiac hypertrophy by working as a transcription cofactor to Nkx2-5 in activating ANF expression (Song et al. 2006).

Protein–protein interaction-based library screen revealed that *Arabidopsis* WRKY transcription factor AtWRKY7 binds to CaM. The CaMBD of AtWRKY7 is conserved among group IID of the WRKY protein family, and all the tested ten WRKY IId members including WRKY 7, 11, 17 bind to calmodulin in a Ca^{2+}-dependent manner (Park et al. 2005). WRKY7 recognizes the conserved W-box (TTGAC) element and acts as a transcriptional repressor in plant cells. WRKY7 responds to pathogen and salicylic acid treatments, and decreased expression of WRKY7 resulted from T-DNA knockout or RNAi-mediated gene silencing correlated with sensitized PR1 induction and enhanced resistance to a virulent *P. syringae* strain. Transgenic plants overexpressing WRKY7 have altered leaf growth and morphology reminiscent of eds8 mutants which are more sensitive to *P. syringae* than wild-type plants. (Kim et al. 2006). Functional analyses also revealed that loss of *WRKY11* in *Arabidopsis* increased the resistance of mutant plants to avirulent and virulent strains of *P. syringae*, and resistant phenotype was further enhanced in the wrky11, wrky17 double mutants, indicating that *WRKY11* and *WRKY17* act as negative regulators of basal immunity. Analyses of transcriptom and expression profiles of selected genes in single and double mutants revealed that both transcription factors modulate transcriptional changes in response to pathogen challenges, in a partially redundant manner (Journot-Catalino et al. 2006). However, whether and how calmodulin regulates the function of the IID class WRKY remain to be addressed.

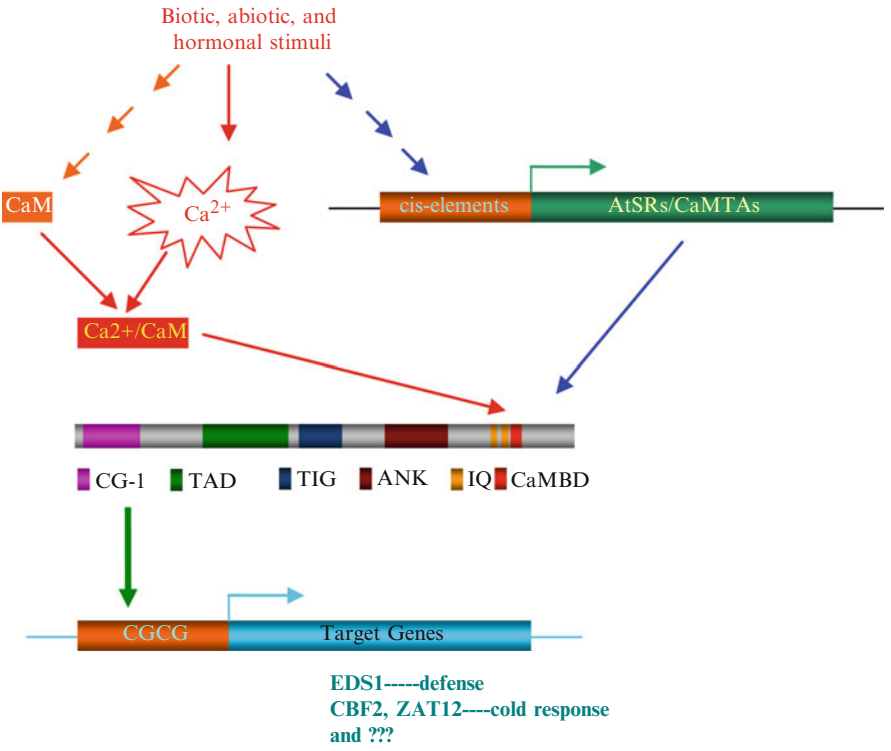

Fig. 5 Schematic illustration of a regulatory model describing the transcriptional control of target genes through the actions of Ca^{2+}, CaM, and AtSRs/CaMTAs. The environmental stimuli are documented to impact the expression of calmodulin, AtSRs/CaMTAs, and also induce transient changes in intracellular Ca^{2+} concentration. These extracellular signals could be integrated into the regulation of AtSR protein, the best characterized CaM-binding transcription factors. AtSRs differentially perceive and respond to a variety of signals and regulate the expression of downstream genes by recognizing the CGCG cis-elements, resulting in upregulated or downregulated expression of the downstream genes and an appropriate physiological response. The *question marks* indicate some unknown targets, and three *short arrows* represent multiple-step processes. CG-1: DNA-binding domain; TAD: transcription activation domain; TIG: nonspecific DNA-binding domain; ANK: ankyrin repeats; IQ: IQ motif; CaMBD: CaM-binding domain

AtMYB2, a transcription factor involved in regulating salt- and dehydration-responses from *Arabidopsis* (Yoshiba et al. 1999; Abe et al. 2003), was found to interact with GmCaM1 and GmCaM4, two different calmodulin isoforms from soybean. The interaction of AtMYB2 with GmCaM4 and GmCaM1 was found to enhance and inhibit its DNA binding in vitro, respectively. Consistently, ecotypic expression of GmCaM4 and AtMYB2 remarkably enhances expression of target genes driven by promoters carrying AtMYB2 recognition motifs (PyAACPyPu), including the proline-synthesizing P5CS1 (delta-pyrroline-5-carboxylate synthetase-1), which confers salt tolerance by facilitating proline accumulation, whereas ecotypic expression of GmCaM1 and AtMYB2 slightly suppresses expression of

AtMYB2 targets in *Arabidopsis* protoplast. As a result, ecotypic expression of GmCaM4 in *Arabidopsis* resulted in elevated tolerance to salt stress in the transgenic plant. Although the functional ortholog of GmCaM4 in *Arabidopsis* was not identified, these results support that the salt- and dehydration-responsive AtMYB2 could be differentially regulated by different calmodulin isoforms (Yoo et al. 2005).

Screening of an *Arabidopsis* cDNA expression library using Hrp-conjugated calmodulin identified a calmodulin-binding NAC domain transcription factor (CBNAC). CBNAC was found to bind to a conserved DNA motif containing a GCTT core sequence. CBNAC bound to a synthetic promoter carrying this recognition motif and repressed the transcription of the associated GUS gene expression in a transient assay using *Arabidopsis* protoplasts, and this repression is enhanced by CaM (Kim et al. 2007). There are over a 100 NAC transcription factors in the *Arabidopsis* genome. NAC members are reported to be involved in developmental regulation and responses to biotic and abiotic stresses (Olsen et al. 2005), but the particular physiological function of CBNAC is not known yet.

3.2.4 CaM Binds Directly to Promoter Element and Regulate Transcription of Target Gene

Although it is generally accepted that calmodulin carries no functional domain besides its four Ca^{2+}-binding EF hands and no enzymatic functions were recorded for the yet multifunctional regulatory protein, a recent development showed that the most conserved isoform of calmodulin in *Arabidopsis*, CaM7, could act as a DNA-binding transcription factor. CaM7 was shown to recognize Z-/G-box light responsive elements in vitro and bind to Z-box containing CAB1 promoter in vivo. Overexpression of CaM7 causes hyperphotomorphogenic growth correlated with increased expression of light-inducible genes. Although null mutants of cam7 grow like wild-type, expression of light-inducible genes was decreased in cam7 mutant, and cam7 hy5 double mutants exhibited an exacerbated phenotype of hy5, a basic leucine zipper transcription factor regulating photomorphogenesis (Jiao et al. 2007). On the other hand, overexpression of CAM7 can partly suppress the hy5 phenotype. These results suggested that CaM7 and HY5 acting as independent transcription factors control the photomorphogenesis in *Arabidopsis* through Z-/G-box LREs in the promoters of light responsive genes (Kushwaha et al. 2008).

In addition to the above-mentioned approaches, other options for Ca^{2+}/calmodulin to regulate transcription in plants cannot be excluded. PCBP is a plant-specific calmodulin-binding protein containing several PEST motifs and exists as a single copy in potato. PCBP is differentially expressed in all of the 12 tested tissues. PCBP targets the nucleus and could be involved in nuclear functions including transcription, although this remains to be verified (Reddy et al. 2002). *Arabidopsis* DRL plays a critical role in controlling meristem activity and organ growth in plants. DRL is a homolog of yeast TOT4/KTI12 with a calcium-dependent calmodulin-binding property. Yeast TOT4/KTI12 associates with Elongator, a complex involved in RNA elongation through the action of RNA polymerase II (Nelissen

et al. 2003). AtCaMBP25 is CaM-binding nuclear protein of 25 kDa isolated from a cDNA expression library derived from *A. thaliana* cell suspension cultures challenged with osmotic stress. AtCaMBP25 is a single-copy gene, and is induced by dehydration, low temperature, or high salinity. AtCaMBP25 is a nuclear protein and acts as a negative regulator of tolerance to osmotic stress. Whether and how AtCaMBP25 is involved in transcriptional control are not addressed (Perruc et al. 2004).

4 Ca^{2+}/CaM-Mediated Regulation of Ion Channel, Transporters, Exchanger, and Other Membrane Proteins

It has been well documented that Ca^{2+}/CaM-mediated signaling plays an active role in regulating both Ca^{2+} influxes and effluxes through the action of Ca^{2+}-permeable channels and Ca^{2+} transporters in animal cells, and similar regulation also exists in plants (Sze et al. 2000). CNGCs are a class of Ca^{2+}-permeable cation channel located at the cytoplasmic membrane and gated by cyclic nucleotide ligands (Kaplan et al. 2007; Ma et al. 2009). Structurally, plant CNGCs carry six transmembrane helices and a CaMBD in the C-terminal extension overlapping a portion of the cyclic nucleotide-binding domain (CNBD), indicating a competitive relationship between cNMP and Ca^{2+}/CaM binding to CNGC channels. Indeed, Hua et al. found that AtCaM4 bound to a CNGC2 fusion protein in a Ca^{2+}-dependent manner with a Kd of 7.6 nM; furthermore, CaM reversed cAMP activation of AtCNGC2 currents in HEK cell (Hua et al. 2003a). The function of AtCNGC10 as a K^+ channel was also found to be regulated by both cGMP and calmodulin in a heterologous complementation tests in *E. coli* K^+ uptake deficient strain LB650. Consistently, Ca^{2+}/CaM was found to counteract the activation of AtCNGC10 function by cGMP (Li et al. 2005). It appears that the physical interaction of Ca^{2+}/CaM with plant CNGCs blocks cyclic nucleotide activation of these channels. Thus, the cytosolic secondary messengers CaM, cAMP, and Ca^{2+} can act in an integrated fashion to gate currents through these plant ion channels. Plant CNGCs have been reported from barley, tobacco, and *Arabidopsis* (Kaplan et al. 2007), and were reported to be involved in various physiological responses including, gravitropic growth of roots (Ma et al. 2006), pollen tube growth (Frietsch et al. 2007), ion homeostasis, transportation and tolerance to heavy metal toxicity (Arazi et al. 1999; Sunkar et al. 2000; Gobert et al. 2006), and programmed cell death and plant immune responses (Clough et al. 2000; Jirage et al. 2001; Balague et al. 2003; Yoshioka et al. 2006; Ali et al. 2007).

Ca^{2+} pumps are important players used to efflux Ca^{2+} from the cytosol, and elevated expression of animal plasma membrane and the endoplasmic reticulum Ca^{2+} pumps (PMCA and SERCA) was reported to change Ca^{2+} signaling kinetics (Brini et al. 2000). Autoinhibited calcium ATPases (ACAs) are the P2B-type (corresponding to animal PM-type) calcium pumps in plants. Although both ACAs from plants and PMCAs from animals are regulated by Ca^{2+}/CaM, there is

an obvious difference between ACAs and PMCAs in that a CaMBD is located in the C-terminal region of PMCA and N-terminus of ACAs (Boursiac and Harper 2007). There are ten homologs of ACAs in *Arabidopsis*, and all share considerable high similarities in both overall sequence and structural features (Axelsen and Palmgren 2001). A regulatory domain is in the N-terminal region which carries a CaMBD and is able to block the Ca^{2+} pump activity, and the bulk of the ACA is made of ten transmembrane helices with small cytoplasmic loop between TM2 and TM3 and big cytoplasmic loop between TM 4 and 5. In contrast to exclusive plasma membrane localization of animal PMCA, the *Arabidopsis* ACAs are located at the plasma membrane (ACA8, 9, 10), ER (ACA2), and vacuolar membrane (ACA4) (Boursiac and Harper 2007). The biophysical function of ACAs as Ca^{2+} pumps was well characterized using complementation tests of yeast strain K616 which are unable to grow on a media with limited supply of Ca^{2+}. Structure–function dissection of *Arabidopsis* ACA8 revealed that the autoinhibition of ACA8 Ca^{2+} pump activity is maintained through intramolecular interaction between the N-terminal regulation region and cytoplasmic loops located between the second and third transmembrane helix of ACA8 (Luoni et al. 2004). Ca^{2+}/calmodulin binding to the N-terminal CaMBD activates ACA8 through an interruption of the inhibitory intramolecular interaction (Beakgaard et al. 2006) and therefore provides a mechanism for feedback regulation on cytosolic Ca^{2+} concentration. Besides regulation by Ca^{2+}/CaM, ACAs could also be regulated by other signaling components such as CDPK (Hwang et al. 2000), acidic phospholipids (Fusca et al. 2009), and possibly by 14-3-3 protein (Palmgren 2003; Rimessi et al. 2005). Because of potential functional redundancy between different ACA homologs, such as ACA8 and ACA10 (George et al. 2008), physiological functions of ACA homologs are just beginning to appear. Independent knockout mutants of ACA9 resulting from T-DNA insertion showed the same phenotype with defects in pollen tube elongation and remarkably decreased fertility in the mutant (Schiott et al. 2004). The gene whose mutation associates with compact inflorescence (cif1) phenotype was recently identified to code for ACA10, and overexpression of both ACA10 and ACA8 could rescue the compact inflorescence phenotype which resulted from the loss of endogenous CIF1 (ACA10) in the presence of a unidentified dominant CIF2 (George et al. 2008).

Human cation/proton exchanger NHE1 was reported to be regulated by Ca^{2+}/CaM-mediated regulations over a decade ago (Bertrand et al. 1994), and similar regulation was recently reported for *Arabidopsis* cation/proton exchanger AtNHX1 by AtCaM15, a variant isoform of calmodulin (Yamaguchi et al. 2005). AtNHX1is the most abundant vacuolar Na^+/H^+ antiporter in *A. thaliana* involved in selective movement of cation between cytoplasm and vacuole. It was reported to affect cellular pH, ion homeostasis, protein trafficking, and plant tolerance to salts (Apse et al. 1999; Bowers et al. 2000). Calmodulin-like protein 15 (AtCaM15) was identified to be an interaction partner of AtNHX1; the interaction between AtCaM15 and AtNHX1 was found to depend on Ca^{2+} and pH, and binding of AtCaM15 to the C-terminus of AtNHX1 decreased its selective exchange of Na^+/H^+ over K^+/H^+, therefore modifying the Na^+/K^+ selectivity of the antiporter (Yamaguchi et al. 2005).

In addition to the above-mentioned ion channels, transporters and antiporters, there are other CaM-binding membrane proteins, such as MLO (Kim et al. 2002b) and apyrase (Steinebrunner et al. 2000). Barley MLO is a plasma membrane protein reminiscent of G protein coupled receptors in animals and is required for powdery mildew fungi to enter the host cell; as a result, mlo null mutants exhibit broad-spectrum resistance to biotrophic pathogens of powdery mildew species (Panstruga 2005). Calmodulin binding to MLO is conducive for MLO to serve as effective entry portals for powdery mildew fungi (Kim et al. 2002a). Apyrase hydrolyzes nucleoside tri- and diphosphates. There are two ecto-apyrases in the *Arabidopsis* genome. Single knockouts of APY1 or APY2 have discernable phenotypic differences as compared to wild-type, double knockout of apy1 and apy2 result in severe defects in growth and pollen germination (Steinebrunner et al. 2003; Wu et al. 2007). Although two apyrases share considerable degree of functional redundancy, only AtAPY1 binds to calmodulin (Steinebrunner et al. 2000). How calmodulin regulates apyrase in plants deserves further attention.

5 Molecular Motors and Their Regulation by Ca^{2+}/Calmodulin in Plants

The high degree of spatial and temporal organization of molecules and organelles is made possible by force-generating protein machines known as molecular motors that transport molecules and organelles to various locations in the cells. Molecular motors are ATPases and move in a specific direction in nanometer steps along the cytoskeleton. Molecular motors can be grouped broadly into three categories: myosins that move along actin cytoskeleton, kinesins that move from the minus end to the plus end of microtubules, and dyneins that move from the plus end to the minus end of microtubules. These motors, in general, help in the delivery of specific cargos that include membranous organelles, protein complexes, and RNAs (Vale and Milligan 2000; Goldstein 2001; Schliwa and Woehlke 2003; Vale 2003). Intracellular transportation is thus fundamental to cellular morphogenesis, development, and physiology of all biological systems.

The basic principles of design of molecular motors and their mechanisms of their movement are well understood (Schliwa and Woehlke 2003; Vale 2003). However an understanding of how they are regulated in cells and their specific functions in cellular physiology is only emerging. In general, molecular motors consist of a globular motor domain and a tail domain. The globular motor domain binds ATP and directly interacts with cytoskeletal tracks. Isolated motor domains can produce movements in vitro. The tail domain corresponds to the rest of the motor. The tail domain is involved in interactions with light chains and cargos as well as in regulating motor activity. Motors are involved in dimerization and formation of higher order structures as in the case of filament formation by polymerization of muscle myosin. During one cycle of the ATP hydrolysis, the motor undergoes a

conformational change that produces force and unidirectional motion. For example, kinesin takes an 8-nm step on microtubule tracks for each ATP hydrolysis (Svoboda et al. 1993; Coy et al. 1999).

Recent advances in genomics and transcriptomics projects have uncovered complete inventories of molecular motors in several organisms. Below we discuss recent understanding of molecular motors in plants and their regulation by calcium and calmodulin.

5.1 Plant Molecular Motors and Cytoskeleton

The identification and characterization of molecular motors has improved our understanding of how plants generate intracellular motility (Asada and Collings 1997; Reddy 2001b). The most prominent form of intracellular motility in plants is the bulk movement of organelles in interphase cells, known as cytoplasmic streaming (Shimmen and Yokota 2004; Verchot-Lubicz and Goldstein 2010), which is dependent on the interaction of organelles with the actin cytoskeleton.

5.2 Myosin Family

5.2.1 Overview

The term "myosin" was originally used to describe a group of ATPases found in striated and smooth muscles (Pollard and Korn 1973). All eukaryotic cells contain myosin isoforms, but some have highly specialized functions (for example in muscle cells) and some are ubiquitous. The head domain binds to filamentous actin while the tail domain interacts with cargos or with other myosin molecules. Myosin moves in an ATP-dependent manner on the actin cytoskeleton from the minus end to the plus end. Myosin VI is an exception to this rule and moves toward the minus end.

There are 20 different classes of myosin, based on amino acid sequences of their ATP hydrolyzing catalytic domain, of which myosin II is the best characterized (Molloy and Veigel 2003). Myosin II moves by converting small structural rearrangements in the catalytic site into a large swing or power stroke of the tail domain.

5.2.2 Plant Myosins and Their Function

Plants express large number of myosins. Vascular plant myosins fall into two classes, namely VIII and XI. *Arabidopsis* encodes 17 myosin genes, four in class VIII and 13 in class XI (Reddy and Day 2000; Lee and Liu 2004). Rice encodes 14

genes, two in class VIII and 12 in class XI (Jiang and Ramachandran 2004). Specific functions of these myosins are poorly understood. Based on immunolocalization data, it is assumed that class VIII myosins act at the plasma membrane (Reichelt et al. 1999; Baluska et al. 2001) and are involved in endocytosis (Sattarzadeh et al. 2008). Class XI myosins have a domain structure similar to class V myosins, which are known to drive vesicle movements in yeasts and mammals.

The actomyosin system has long been implicated in cytoplasmic streaming in plant cells. For example, cytoplasmic streaming in pollen tubes is supported by the actomysin system (Kohno and Shimmen 1988; Cai and Cresti 2009). Actomyosin motility of endoplasmic reticulum and chloroplasts in Vallisneria mesophyll cells are sensitive to disruption by the actin depolymerizing drug cytochalasin D and thapsigargin, an inhibitor of the ER Ca^{2+}-ATPase (Liebe and Menzel 1995).

Knockout mutant studies in *Arabidopsis* have defined specific function for class XI myosins. They are involved in organellar trafficking and development of root hair. Interestingly, inactivation of class II myosin caused no detectable phenotypes in *Arabidopsis* (Peremyslov et al. 2008; Prokhnevsky et al. 2008).

5.2.3 Regulation

Calcium is a negative regulator of cytoplasmic streaming (Nakamura and Kohama 1999; Yokota et al. 1999) and is the overwhelming mode of regulation of cytoplasmic streaming of plant cells and lower eukaryotes. It is not clear how Ca^{2+} regulates class XI family of myosins in plants. Activity of animal myosin V, a member of the XI family, is directly regulated by Ca^{2+} (Nguyen and Higuchi 2005). An increase in Ca^{2+} levels to 1 μM is correlated with a reduction of sliding velocity where as 5 μM Ca^{2+} results in the detachment of actin filaments from myosin V.

Activity of motor protein may be controlled by regulating cytoskeletal properties, by regulating motor proteins by covalent modifications, or by motor interacting proteins. In a recent study (Del Duca et al. 2009) pollen transglutaminases appear to control different properties of the pollen tube cytoskeleton and its interactions with motor proteins, and they are critical for the development of pollen tubes.

5.2.4 Chara Myosin

Myosin motor in algae *Chara* deserves special mention because it is the fastest known motor protein. In the giant cells of Characeae, the cytoplasm streams very rapidly rotationally along chloroplasts just beneath the cell wall. This cytoplasmic streaming reported by Kamiya and Kuroda (1956) is dependent on the actomyosin system (Kamiya and Kuroda 1956). Chara myosin belongs to the class II myosin family and surprisingly this myosin exhibits the fastest sliding movement of F-actin at 60 uM/S in the in vitro motility assays (Higashi-Fujime and Nakamura 2009).

This velocity is approximately ten times greater than myosin from skeletal muscles (Higashi-Fujime et al. 1995; Rivolta et al. 1995; Yamamoto et al. 1995). Chara myosin did not polymerize into filaments, but it transported vesicles and this motility is regulated by phosphorylation (Morimatsu et al. 2002).

5.3 Dynein Family

5.3.1 Overview

Cytoplasmic dynein is the most complex and least understood molecular motor. Dynein is a large protein complex (1.2 MDa) containing two identical heavy chains (<500 KDa) and several intermediate and light chains (Vallee et al. 2004). The heavy chain has three functionally distinct domains: a globular head with ATPase activity, a cargo-binding tail, and a microtubule-binding stalk. The tail is formed by the N-terminus of the heavy chain. It associates with accessory proteins and binds various cargos. An N-terminus region of the head domain is called the "linker" domain, and its interaction with the dynein head is thought to be important for power generation (Kon et al. 2005; Reck-Peterson et al. 2006).

The head makes up two-thirds of the C-terminal heavy chain and consists of six AAA + domains (AAA1–6) in a ring-like arrangement. High resolution electron microscopy with image averaging indicates a heptameric wheel-like structure of the dynein motor domain, with the six AAA domains plus an additional C-terminal domain. The first four domains each contains a P-loop known to be important for ATPase activity (Mocz and Gibbons 1996). The head also contains an additional domain (418 residues) at the C-terminus, the function of which is still unknown (Burgess et al. 2003; Samso and Koonce 2004). A long stalk (10–15 nm) protrudes from the head (Goodenough and Heuser 1982) and comprises a coiled-coil domain ending in a small globular domain that binds microtubules in an ATP-dependent manner (Gee et al. 1997; Mizuno et al. 2004).

Cytoplasmic dynein complexes participate in many intracellular transport activities in eukaryotes, such as localization of mRNAs, transport of intermediate filament and centrosomal proteins, nuclear envelope breakdown, apoptosis, virus transport, and functioning of kinetochore (Hirokawa 1998; Vale 2003).

5.3.2 Plant Dyneins and Their Function

The cytoplasmic dynein has been implicated in a wide variety of essential transport activities, including vesicle mobility, retrograde transport, and mitosis, and it also provides the power for ciliary and flagellar motility. In mammals, null mutants of dynein die early in embryogenesis.

Many algae such as Chlamydomonas and Volvox and the spermatoids of the water fern Marsilla contain motile flagella which are powered by dyneins

(King 2002). With the availability of shotgun sequences of the rice genome, the presence of four dynein heavy chains has been revealed (King 2002). Surprisingly, dynein-like sequences were not found in the genome sequences of *Arabidopsis* (Lawrence et al. 2001). With the availability of genome sequences of more plants, we will be able to examine the evolution of dynein system in plants. Proteins that cross-react with dynein heavy chain antiserum have been reported in pollen tubes of *N. tabacum* (Moscatelli et al. 1995) and *Ginko biloba* (Moscatelli et al. 1996). Recently, nucleotide sequences corresponding to dynein heavy chain have been isolated from Nicotiana (Scali et al. 2003) and from Avicennia (Moscatelli et al. 2003). Other members of the complex such as intermediate chains and light chains remain to be characterized. Though dynein has been implicated in cytoplasmic streaming and cargo transport in plants, the specific functions of the dynein system in plant growth and development remain to be explored.

5.3.3 Regulation

Calcium/calmodulin appears to play a crucial role in regulating ciliary and flagellar dynein. In 1980, the Vanaman group reported that the 14 S and 30 S dyneins of Tetrahymena contain Ca^{2+}-dependent binding sites for calmodulin (Blum et al. 1980). The cytoplasmic dynein of sea urchin directly interacts with calmodulin (Hisanaga and Sakai 1983). In fact sea urchin dynein can be purified using a calmodulin-sepharose column.

Ca^{2+} induces an increase in dynein activity in *Chlamydomonas axonemes* and this increase is blocked by inhibitors of calmodulin and calcium/calmodulin-dependent protein kinase (Smith 2002). Calmodulin is localized to axonemes and play as a key calcium sensor (Dymek and Smith 2007). Two IQ consensus motifs for binding calmodulin-like proteins are located within the stem domain of the Chlamydomonas gamma heavy chain (Sakato et al. 2007). In vitro experiments have shown that in the presence of Ca^{2+}, dynein light chain (LC4) binds to the IQ motif of heavy chain. Ca^{2+} binding also causes the dynein complex to adopt a more compact structure (Sakato et al. 2007).

5.4 Kinesin Family

5.4.1 Overview

The kinesin family of molecular motors moves along microtubules using ATP as its energy source. The kinesin motor complex contains kinesin heavy chains and light chains. Heavy chains contain the motor domain that facilitates movement. The light chains interact with heavy chains and assist in cargo transport. Heavy chains and light chains may interact with different protein cargos.

Depending on the position of the motor domain in the kinesin-heavy chain, kinesins are grouped into three families: N-terminal, C-terminal, and intermediate

type. Kinesins are further grouped into more than a dozen subfamilies by phylogenetic analysis of the motor domains (Schoch et al. 2003; Dagenbach and Endow 2004). According to a new nomenclature system proposed by the kinesin research community (Lawrence et al. 2004), kinesin subfamilies are classified from kinesin 1 to kinesin 14.

Kinesins in general move from the minus end to the plus end of microtubules. However members of kinesin C or C-terminal kinesins move toward the minus end. Kinesins are involved in a wide range of functions, i.e., for chromosome attachment to microtubules, chromatid segregation, spindle elongation (Sharp et al. 2000), vesicles and organellar transport (Vale 2003; Wozniak et al. 2004; Hirokawa et al. 2009), RNA transport (Kanai et al. 2004), and in memory storage (Puthanveettil et al. 2008).

5.4.2 Plant Kinesins and Their Function

Kinesins are by far the most well-studied molecular motors in plants. Though the exact functions of the plant kinesins are poorly understood, plant genomes seem to possess the ability to encode large number of kinesins. For example, the completed genome of *Arabidopsis* contains 61 genes encoding polypeptides with the kinesin catalytic core (Reddy and Day 2001). Based on the signature "neck sequence" (Endow 1999) there are 21 *Arabidopsis* genes encoding minus end-directed kinesins (Reddy and Day 2001). These seven kinesins contain a unique calponin-homology (CH) domain at the N-terminus. The presence of this CH domain confers the ability to interact with actin (Gimona et al. 2002; Korenbaum and Rivero 2002). These kinds of kinesins are so far only reported in plants and are called KCH (kinesins with a CH domain) proteins. Functions of these KCH proteins are not understood. Four of the minus end-directed kinesins contain motor domains at the N-terminus. Interestingly, such a feature is not reported in animals and fungi.

Calmodulin-binding kinesin is another member of the minus end-directed kinesin. *Arabidopsis* KCBP/ZW1 kinesin is a calmodulin-binding kinesin (Reddy and Day 2001) and loss-of-function mutations cause a reduction of branch formations of leaf trichomes (Oppenheimer et al. 1997). Several of the interacting proteins of KCBP (Day et al. 2000; Folkers et al. 2002; Reddy et al. 2004) play a role in trichome branching as well. It has also been suggested that KCBP functions as microtubule stabilizer during cell morphogenesis (Mathur and Chua 2000; Preuss et al. 2003). KCBP is differentially regulated during cell division in Tradescantia (Vos et al. 2000). Injection of antibodies to stimulate KCBP during late prophase caused nuclear envelope breakdown and resulted in the arrest of mitosis. Cells did not progress into anaphase. However injection of these antibodies during later phases of cell division did not affect anaphase transition but delayed the completion of cytokinesis.

Arabidopsis KATA/ATK1 kinesin is a nonprocessive minus end-directed motor and plays a critical role in the microtubule organization at the spindle pole and spindle midzone during meiosis. Loss-of-function mutations cause abnormal

chromosomal segregation during microsporogenesis (Chen et al. 2002). Similarly *atk1-1* mutation causes abnormalities in microtubule accumulation at the spindle poles during the early stages of mitotic spindle assembly (Marcus et al. 2003). However this does not affect the ultimate outcome of mitosis (Marcus et al. 2003).

Besides these minus end-directed kinesins, the *Arabidopsis* genome encodes the N-terminal motor kinesin AtFRA1, two internal motor kinesins (At3g16630, 16060), and four genes (At2g28620,36200,37420,45850) belonging to BIMC/Kinesin5 subfamily (Reddy and Day 2001). AtFRA1 was identified by a screening for fragile fiber mutants (Zhong et al. 2002). These mutants displayed altered orientation of cellulose microfibrils in fibers of the inflorescence stems.

5.4.3 Regulation

One of the best studied plant kinesins is *K*inesin-like *C*almodulin-*b*inding *P*rotein (KCBP). KCBP was originally isolated and characterized from plants by the Reddy and Poovaiah laboratories (Reddy et al. 1996; Wang et al. 1996; Bowser and Reddy 1997; Narasimhulu et al. 1997; Song et al. 1997; Reddy and Reddy 1999). KCBP is also expressed in monocots (Abdel-Ghany and Reddy 2000; Abdel-Ghany et al. 2005) and algae unicellular flagellate Chlamydomonas (Dymek et al. 2006). Interestingly, KCBP is also reported in sea urchin (Rogers et al. 1999).

KCBP is encoded by a single gene in *Arabidopsis* and highly expressed in developing flowers and cell suspension cultures. KCBP is a minus end-directed kinesin and belongs to the Kinesin-14 family. Its calmodulin-binding domain is regulatory and is adjacent to the C-terminal motor domain (Reddy et al. 1996; Narasimhulu and Reddy 1998). Binding of Ca^{2+}/calmodulin to KCBP causes the motor to dissociate from the microtubule cytoskeleton (Deavours et al. 1998). Thus Ca^{2+}/calmodulin functions as a negative regulator of KCBP. X-ray crystallographic studies of KCBP have revealed that the calmodulin-binding domain is positioned relative to the globular motor core (Vinogradova et al. 2004, 2008). According to the crystal structures of KCBP, binding of Ca^{2+}/calmodulin to the regulatory helix would sterically block the microtubule binding sites of KCBP and prevents KCBP from attaching to microtubules. Using this knowledge, Konishi et al (2006) engineered a kinesin with a Ca^{2+}-dependent switch such that the engineered kinesin is switched on/off reversibly by externally delivered Ca^{2+}/calmodulin (Konishi et al. 2006).

6 Cross Talk with Other Signal Transduction Pathways

Calmodulin-binding proteins are often present at the crossroads with other signaling pathways, such as those involving reactive oxygen species (ROS), phospholipid messengers, and ethylene, brassinosteroids, salicylic acid, and MAP kinase pathways. These proteins are possible candidates for the signal nodes connecting

to and coordinating with the different signal networks in plants. Here we will focus on the cross talk with oxidative signaling, GABA pathway, auxin signaling, brassinosteroid signaling, and phospholipid signaling since others have been addressed in other portions.

6.1 Oxidative Signaling

Reactive oxygen species (ROS) are a collective term for radicals derived from O_2, such as hydrogen peroxide, superoxide anion, and hydroxyle radicals, which are produced during normal metabolism and plant response to abiotic and biotic stresses (Levine et al. 1994; Prasad et al. 1994; Van Breusegem et al. 2001). ROS play two divergent roles: exacerbating damage and signaling the activation of defense responses under biotic and abiotic stresses. H_2O_2 is beginning to be accepted as a second messenger for signals generated by means of ROS because of its relatively long life and high permeability across membranes. To allow for these different roles, cellular levels of ROS must be stringently controlled. Recent studies suggest that changes in intracellular redox and calcium homeostasis are unifying consequences of biotic and abiotic stresses.

Treatment with H_2O_2 can stimulate increases in cytosolic Ca^{2+} by activating the calcium channel (Price et al. 1994). Nonetheless, H_2O_2 production in the oxidative burst requires a continuous Ca^{2+} influx, which activates the plasma membrane-localized NADPH oxidase (Xing et al. 1997). Ca^{2+}-binding EF hands are present in the $gp91^{phox}$ subunit of NADPH oxidase (Keller et al. 1998). Ca^{2+}/CaM has been proposed to increase H_2O_2 generation through Ca^{2+}/CaM-dependent NAD kinase that affects the concentration of available NADPH during assembly and activation of NADPH oxidase (Harding et al. 1997). Two plant NAD kinases have been cloned, and calmodulin can bind one of them (Turner et al. 2004). Moreover, Ca^{2+}/CaM binds to plant catalase and enhances its activity. Catalase, which degrades H_2O_2 into water and oxygen, is one of the major antioxidant enzyme scavengers and plays an important role in plant defense, aging, and senescence. Yang and Poovaiah isolated an *Arabidopsis* catalase isoform (AtCat3) which can be bound by CaM in a Ca^{2+}-dependent manner (Yang and Poovaiah 2002b). The activity of the enzyme was stimulated about twofold by Ca^{2+}/CaM, but not by Ca^{2+} or CaM alone. The regulation of catalase by CaM is probably specific to plants because CaM had no effect on catalases from sources such as *Aspergillus niger*, human erythrocytes, and bovine liver. In addition, CaM was detected with specific antibodies in peroxisomes isolated from etiolated pumpkin cotyledons. Peroxisomes are specialized organelles involved in the catabolic oxidation of various biomolecules and the resultant H_2O_2 is consumed by peroxysomal catalases. Another class of ROS-scavenging enzymes, the superoxide dismutases (SODs), was suggested to be calmodulin-binding proteins (Gong et al. 1997). SOD from maize germs was shown to reversibly bind CaM immobilized on a column in a Ca^{2+}-dependent manner. However, these findings were never confirmed in

CaM-binding assays with recombinant SODs or SODs purified from other sources. Recently, *Euphorbia* latex peroxidase was shown to have two distinct amino acid sequences recognized as CaM-binding sites. CaM-binding assays and the determination of steady-state parameters showed unequivocally that *Euphorbia* peroxidase is a CaM-binding protein activated by Ca^{2+}/CaM. These findings suggest that peroxidase might be another node in the Ca^{2+}/H_2O_2-mediated plant defense system, having both positive and negative effects in regulating H_2O_2 homeostasis (Mura et al. 2005). Importantly, the expression of genes encoding calmodulin-binding transcription factors is stimulated by H_2O_2, such as AtBTs and AtSR, which has been discussed in other sections, suggesting the broader impact of calcium/calmodulin on oxidative signaling.

6.2 Aminobutyric Acid and Glutamate Decarboxylase

Aminobutyric acid (GABA) is a four-carbon nonprotein amino acid. In human and mammals, GABA acts as a major inhibitory neurotransmitter (Kazemi and Hoop 1991). The presence of GABA in plants was first described in potato tubers in 1949 (Steward et al. 1949). The production of GABA has been associated with stresses such as anaerobic conditions (Streeter and Thompson 1972), low or high temperatures and darkness, and mechanical manipulation (Wallace et al. 1984; Bouche et al. 2004). The majority of scientific studies carried out with the aim of deciphering the functional roles of GABA have concentrated on stress-response and its signaling roles (Bouche and Fromm 2004), as well as its roles as a metabolite (Bouche and Fromm 2004; Fait et al. 2008).

GABA is synthesized from glutamate and this reaction is catalyzed by GAD (Bouche and Fromm 2004). In plants, petunia GAD was first characterized as a calmodulin-binding protein. GAD activity is essentially upregulated by calcium/calmodulin in physiological pH (Baum et al. 1993, 1996; Snedden et al. 1996). Later the GAD was cloned and characterized in many other plant species, including *Arabidopsis*, tomato, *Vicia faba*, soybean, tobacco, rice, and asparagus (Bouche and Fromm 2004). Almost all of plant GADs have a plant-specific calmodulin-binding domain in the C-terminus, while this feature is not present in any mammalian or *E. coli* GADs, suggesting that the regulation of GAD activity by calcium–calmodulin is plant specific.

The physiological relevance of GAD activation by calmodulin was first addressed in tobacco by using transgenic plants ectopically expressing either GAD or a truncated GAD that could not bind CaM. Removal of the C-terminal calmodulin-binding domain of GAD resulted in constitutive GAD activity (i.e., calcium independent), abnormal steady-state levels of glutamate (low) and GABA (high), and aberrant plant development. Therefore, calcium/calmodulin is critical in controlling the activity of GAD in vivo and this regulation is necessary for normal plant development. In *Arabidopsis*, disrupting the root-specific *GAD1* gene using T-DNA insertion mutagenesis revealed that it plays a major role in GABA synthesis

under normal growth conditions and in response to heat stress (Bouche et al. 2004). Recent crystal structure studies of GAD revealed the mechanisms for calcium/calmodulin-regulation and pH regulation modes and provide a model to explain the rapid stimulation of GAD activity in response to biotic and abiotic stresses, which elicit changes in cytosolic Ca^{2+} concentrations, suggesting that the production of GABA via the stimulation of GAD by calmodulin and cytosolic calcium fluxes may be a unifying event (Gut et al. 2009). Mutation of two crucial residues made the enzyme insensitive to both its normal levels of regulation (pH-dependent and CaM-mediated), demonstrating how these two levels rely on a common molecular basis (relief of autoinhibition).

6.3 *DWF1 and Brassinosteroids*

Brassinosteroids (BRs) are a relatively new class of plant hormones which play critical roles in regulating plant growth, development, and responses to environmental cues such as light. As seen in *Arabidopsis* plants, mutants failing to synthesize or perceive BRs usually show a typical dwarf phenotype and photomorphogenesis in dark conditions. Mutants with elevated endogenous BR levels resulted from overexpression of BR biosynthetic genes, such as DET2, CPD, and DWF4, usually exhibit hypermorphic phenotypes with increased vegetative growth (Bishop and Koncz 2002; Clouse 2002; Nemhauser and Chory 2004). The *Arabidopsis* det3 mutant carries a mutation in the gene coding for subunit C of the vacuolar H^+-ATPase (VHA-C). The det3 mutant is dwarf and exhibited de-etiolated phenotype in dark conditions. This phenotype could be partially rescued by exogenous application of BR (Schumacher et al. 1999). It was discovered later that compared with wild-type, det3 mutant produces altered Ca^{2+} oscillation and guard cell response after various treatments (Allen et al. 2000). These results implied a correlation between calcium and plant growth and development via the action of hormones, such as BRs. In an independent study, upregulated expression of a conserved CaM isoform in potato resulted in increased growth and apical dominance in potato (Poovaiah et al. 1996), indicating that calmodulin-mediated signaling is involved in plant growth. A direct proof for the involvement of Ca^{2+}/CaM-mediated signal in the action of BR was provided by the identification of DWF1 as a calmodulin-binding protein (Du and Poovaiah 2005).

DWF1 is an essential enzyme which converts 24-methylenecholesterol to campesterol during brassinosteroid biosynthesis (Klahre et al. 1998; Choe et al. 1999). Screening of *Arabidopsis* cDNA library using ^{35}S methionine-labeled calmodulin revealed that DWF1 interacts with conserved calmodulin isoforms through its C-terminal CaMBD in a Ca^{2+}-dependent manner, and the interaction between DWF1 and calmodulin in vivo was confirmed using coimmunoprecipitation combined with prior cross-link strategy (Du and Poovaiah 2005). Functional characterization of DWF1-CaM interaction revealed that CaM binding to DWF1 is a necessary posttranslation regulatory mechanism required for its normal function during brassinosteroid biosynthesis (Du and Poovaiah 2005), coinciding with previous

observations that the loss of DWF1 resulted in dwarfism and overexpression of DWF1 had no phenotypic impact (Klahre et al. 1998). The calmodulin-binding property of DWF1 was conserved among orthologs from other plants but not from animals, indicating that this posttranslation regulation by Ca^{2+}/CaM is common in plants.

Besides DWF1, other brassinosteroid synthetic enzymes such as DWF4 and CPD could also be regulated by Ca^{2+}/CaM (Du and Poovaiah 2005; Yang et al. 2007). DWF4 and CPD are both Cyt P450 enzymes which share a considerable degree of homolog with other CYP90s and CYP85As involved in brassinosteroid synthesis. There are two CaM-binding domains (CaMBDs) in DWF4; one is located between aa 16–40 in the N-terminus overlapping with a transmembrane helix and the other is in the C-terminal region (aa 374–397) overlapping with the subject recognition site 5 (SRS5) with a conserved Glu-X-X-Arg motif. Sequence alignment revealed that the N-terminal CaMBD of DWF4 is not conserved but the C-terminal CaMBD of DWF4 is highly conserved among the Cyt P450 members involved in BR biosynthesis. Gel mobility shift assay of calmodulin and peptide interaction revealed that the N-terminal CaMBD of DWF4 binds to Ca^{2+}/CaM with a significantly higher affinity than the C-terminal CaMBD, and the CaMBD from CPD which shares high homology with C-terminal CaMBD of DWF4 also binds to Ca^{2+}/CaM but with an apparently weaker affinity. An effort to address the functional significance of CaM binding to DWF4 or CPD by molecular genetic approach generated no conclusive results since mutations in the CaMBDs resulted in the loss of DWF4 function regardless of its CaM binding (Yang et al. 2007). However, the overlapping of CaMBD with critical functional domains and the zero tolerance to mutation in these regions, especially the SRS5 of these Cyt P450 proteins, imply a functional role for their calmodulin binding. Enzymatic assay in vitro with purified protein or ectopically expressed protein in yeast cell as described (Waterham et al. 2001) might be better choices to study the regulation of Ca^{2+}/CaM on these Cyt P450s.

Regulation of multiple players including DWF1 and possibly DWF4, CPD, and other Cyt P450 proteins indicate that calcium-mediated signaling might exert a comprehensive control on the biosynthesis of brassinosteroid. A very interesting finding showed that BRI1, the surface receptor of BRs, has guanylyl cyclase activity and could catalyze the production of cGMP (Kwezi et al. 2007), a second messenger regulates CNGC channels and the generation of calcium signal (Ma et al. 2009). Unlike other photohormones, such as auxin, BRs do not undergo a long-distance transportation within the plant (Symons and Reid 2004). Coordinating the action of BRs *in planta* would be very difficult without the interplay of BR and other messenger/signaling system. The discovery of connections between BR actions and Ca^{2+}, an integral part of the cellular signaling system in plants, brings new insight into the coordination of BR actions.

6.4 Auxin Signaling and Small Auxin Up RNAs (SAURs)

Auxin is one of the major hormones in plants and plays a central role in growth and development by controlling cell division, cell elongation, and cell differentiation, as well as plant response to gravity (Benjamins and Scheres 2008). Accumulating

evidence indicates that there is a close relationship between the mechanism of auxin action and calcium signaling (Hardin et al. 1972; Abel and Theologis 1996). The effect of auxin on changes in cellular calcium levels has been obtained using calcium-sensitive fluorescent dyes or Ca^{2+}-sensitive microelectrodes (Evans and Cleland 1985). Felle reported a decrease in free calcium in cells after auxin treatment (Felle 1988), whereas Gehring et al. observed an increase in calcium levels after auxin treatment (Gehring et al. 1990). Depletion of calcium in tissues using calcium chelators and CaM inhibitors has implicated a role for calcium in the auxin signal transduction. Raghothama et al. found that CaM antagonists such as chlorpromazine, trifluoperazine, fluphenazine, and W-7 inhibited the auxin-induced elongation of oat and corn coleoptile segments (Raghothama et al. 1985). Gonzalezdaros et al. observed that some, but not all, calmodulin inhibitors tested could inhibit auxin-induced medium acidification by oat coleoptile segments (Gonzalezdaros et al. 1993). Similarly, Reddy et al. observed that the calcium chelator EGTA and calcium channel blocker D-600 inhibited auxin-induced elongation of pea epicotyl segments (Reddy et al. 1988). Auxin has been linked with calcium transport, release of calcium ion from membrane vesicles, and phosphatidylinositol hydrolysis (Kubowicz et al. 1982).

Yang and Poovaiah reported that the maize auxin early responsive gene ZmSAUR1 encoded a CaM-binding protein (Yang and Poovaiah 2000a). *SAURs* belong to one group of the early auxin-response genes (McClure and Guilfoyle 1989; McClure et al. 1989). Initially isolated from soybean (McClure and Guilfoyle 1987), *SAUR* genes have also been characterized from several dicots such as mung bean, *Arabidopsis*, apple, and corn. In all cases examined, *SAUR* genes encode short transcripts with highly conserved open reading frames that accumulate rapidly and specifically after auxin treatment. Soybean *SAUR* gene transcription can be detected as soon as 2.5 min after the application of auxin (McClure and Guilfoyle 1989; McClure et al. 1989). The calcium/calmodulin binds to a CaM-binding site located at the NH_2-terminal domain. There are 72 SAUR genes in *Arabidopsis* (Park et al. 2007) and 58 SAUR genes in rice (Jain et al. 2006). Comparison of the NH_2-terminal portions of all of the characterized SAURs revealed that they all contain a stretch of the basic α-amphiphilic helix similar to the CaM-binding region of ZmSAUR1. For example, calmodulin binds to the two synthetic peptides from the NH_2-terminal regions of *Arabidopsis* SAUR-AC1 and soybean 10A5, suggesting that this is a general phenomenon for all SAURs. The functions of some of SAURs have been characterized. For example, transgenic *Arabidopsis* seedlings overexpressing a *SAUR* gene abolished apical hook maintenance 1 (*AAM1*) exhibited short, hookless hypocotyls with partially open cotyledons in darkness (Park et al. 2007). The hookless phenotype was completely rescued by exogenous auxin. In contrast, hypocotyls of a T-DNA insertion mutant *aam1* were slightly longer than wild-type hypocotyls and exhibited normal hook curvature. Transgenic rice plants overexpressing the *SAUR39* gene in rice lead to lower shoot and root growth, altered shoot morphology, smaller vascular tissue, and lower yield as compared with wild-type plants (Kant et al. 2009). Biochemical analysis also revealed that transgenic plants had lower chlorophyll content, higher levels of

anthocyanin, abscisic acid, sugar, and starch, and faster leaf senescence compared with wild-type plants at the vegetative stage. Most of these phenomena have been shown to be negatively correlated with auxin level and transport. Transcript profiling revealed that metabolic perturbations in overexpressed plants were largely due to transcriptional changes of genes involved in photosynthesis, senescence, chlorophyll production, anthocyanin accumulation, sugar synthesis, and transport. The lower growth and yield of overexpression plants was largely recovered by exogenous auxin application, suggesting that SAUR acts as a negative regulator for auxin synthesis and transport. However, the importance of calcium/calmodulin binding to SAUR remains to be resolved.

6.5 *Phospholipids Signaling*

Phospholipid molecules are one of the main structural components of membranes which play a key role in maintenance of the requisite physical properties and functionality of cell membranes. In recent years, phospholipids are becoming one of the important second messengers to regulate plant growth and development and cellular responses to environmental change or stress (Wang 2001; Xue et al. 2007). An increase in membrane permeability during stress and senescence in part reflects a general decline in calcium-ATPase activities in cell membrane (Paliyath and Thompson 1988). Calcium-ATPase serves to extrude or sequester free cytosolic calcium and thereby maintain the submicromolar calcium concentration required for normal cell function. Recently, ACA8, a plasma membrane calcium-ATPase in *Arabidopsis*, was found to be regulated by phospholipids and calmodulin because the phospholipid-binding site overlaps the calmodulin-binding site (Meneghelli et al. 2008). Acidic phospholipids increased $V_{max\text{-}calcium}$ and lowered the value of $K_{0.5\text{-}calcium}$ below the value measured in the presence of calmodulin. In the presence of calmodulin, acidic phospholipids activated ACA8 by further decreasing its $K_{0.5\text{-}calcium}$ value, suggesting the complex interactions of calcium–calmodulin and phospholipids in plants.

Phospholipid hydrolysis occurs in response to various cellular and environmental cues. Such hydrolysis is involved in many cellular processes through roles in generating signal messengers, cytoskeletal rearrangements, vesicular trafficking, secretion, ion fluxes, membrane remodeling, and lipid degradation. Phospholipases are key enzymes catalyzing the initial step of lipid hydrolysis. These enzymes are grouped into four major classes: phospholipase D (PLD), PLC, PLA_2, and PLA_1, according to the site of lipid hydrolysis. It is well known that PLD is activated by Ca^{2+} via the calcium binding to the C2 domain (Pappan and Wang 1999). Calcium-activated PLD binds to cell membranes and cleaves phospholipids yielding phosphatidic acid (PA). In turn, PA has been identified as an important lipid second messenger involved in responses to hormones and various types of stress (Wang 2000, 2002, 2005; Bargmann and Munnik 2006; Wang et al. 2006). PA cleaved to diacylglycerol (DAG), which can also serve as a lipid messenger, by an acidic

phosphatase that is regulated by calmodulin (Paliyath and Thompson 1987). Snedden and Blumwald (2000) reported that calmodulin binds to one of two tomato diacylglycerol kinases (DGKs) (Snedden and Blumwald 2000). DGKs catalyze phosphorylation of diacylglycerol (DAG) to yield phosphatidic acid (PA). The two enzymes are derived from the same gene via alternative splicing and are identical except for a 29 residue C-terminal extension of LeCBDGK identified as a calmodulin-binding domain. These findings suggest a mechanism whereby DGKs may directly couple Ca^{2+} and phospholipid signaling in plants.

6.6 MAPK Pathways and MPK Phosphatase

Mitogen-activated protein kinase (MAPK) cascades are an important means of signal transduction in plants and other eukaryotes, linking perception of an environmental or developmental signal to downstream targets. The MAPK signaling consists of a linear cascade of three consecutively acting protein kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK). Plants possess multiple MAPKKKs, MAPKKs, and MAPKs, which respond to different upstream signals and activate distinct downstream pathways (Tena et al. 2001; Nakagami et al. 2005; Colcombet and Hirt 2008). The phosphorylated (activated) MAPK interacts with and alters the phosphorylation status of target proteins, including transcription factors, enzymes, and other proteins, ultimately influencing gene expression, metabolism, cell division, and growth.

Another stress-activated protein phosphorylation is mediated by calcium-dependent protein kinases. It has been reported that a calcium-dependent protein kinase, NtCDPK2, cross talks with MAPK signaling using ethylene as a link (Ludwig et al. 2005). While there is still no direct evidence of calmodulin involvement in MAPK-mediated phosphorylation signaling in plants, several lines of evidence indicate that calmodulin regulates the MAPK dephosphorylation via binding to MAPK phsophatase (Yamakawa et al. 2004; Katou et al. 2005, 2007; Chen et al. 2007; Rainaldi et al. 2007; Lee et al. 2008; Ishida et al. 2009; Yoshioka et al. 2009). The regulation of dephosphorylation and inactivation of MAPKs by protein phosphatases is a critical component of MAPK signaling (Ulm et al. 2002; Bartels et al. 2009). A tobacco MAPK phosphatase gene, NtMKP1, was first defined as a candidate gene for a calmodulin-binding protein (Yamakawa et al. 2004). The bacterially expressed NtMKP1 protein physically interacted with three plant-specific types of calmodulin. In comparison to the transient accumulation of a wound-induced MAPK/WIPK transcript, a prolonged activation of NtMKP1 expression was found in response to wounding and tobacco mosaic virus-induced hypersensitive reaction. In transgenic tobacco plants overexpressing NtMKP1, wound-induced activation of SIPK, salicylic acid-induced MAPK, and WIPK was inhibited. These results suggest that plant CaMs are involved in these stress-activated MAPK cascades via NtMKP1 (Yamakawa et al. 2004). Later, rice OsMKP1 and *Arabidopsis* AtMKP1 were reported to bind calmodulin. Interestingly, *Arabidopsis*

AtMKP1 has two calmodulin-binding sites (Lee et al. 2008; Ishida et al. 2009). The peptides for these two calmodulin-binding sites bound CaM in a calcium-dependent manner with different affinities. Furthermore, calcium/calmodulin can increase the phosphatase activity of AtMKP1 (Lee et al. 2008) .

Ulm et al. (2002) found that MKP1 interacts with several MAPKs in vitro and provided evidence that MKP1 regulates the activity level of MPK6 *in planta*, especially in relation to abiotic stress responses. Recently, Bartels et al. (2009) reported on the coordinated regulation of the MAPK MPK6 by the phosphatases MAPK phosphatase1 (MKP1) and protein tyrosine phosphatase1 (PTP1) in Arabidopsis and their relation to stress responses (Bartels et al. 2009). A detailed analysis of null *mkp1* and *ptp1* mutations in *Arabidopsis* indicates that the mutations are associated with a deregulation of MPK6 that causes constitutive defense responses and leads to an accumulation of salicylic acid and camalexin and significantly reduced growth. Therefore the negative regulation of the complex MAPK signaling is very important in fine-tuning plant response to environmental stress. Calcium/calmodulin could be a coordinator in this complex process by its regulation of MPK1.

7 Summary and Outlook

It has been observed that many abiotic and biotic stimuli such as heat, cold, drought, salt, light, wind, touch, wounding, symbionts, and pathogens, as well as growth, developmental and hormonal cues can quickly induce cytosolic Ca^{2+} increases (Evans et al. 2001; Reddy 2001a; Snedden and Fromm 2001; Yang and Poovaiah 2003; Bouche et al. 2005). As mentioned, calmodulin, the most thoroughly studied calcium sensor, mediates interpretation of calcium signals in various aspects of plant life including growth and development, reproduction, and responses to biotic and abiotic stresses. Although calmodulin usually does not have its own specific enzymatic/biochemical function, as discussed above, calmodulin was found to interact with a wide spectrum of target proteins which carry various enzymatic/biochemical/physiological functional roles such as kinases, catalytic enzymes, transcription factors, cytoskeleton proteins, and various pump/channel proteins. Therefore, CaM or CML acts as a multifunctional regulatory protein. Since there are multiple calmodulin isoforms and calmodulin-like proteins in a plant genome, identifying additional new target proteins of CaMs and CMLs is still far from the final stage, especially those which interact with CMLs. On the other hand, the calmodulin-mediated regulations of target proteins in plants are frequently presumptive such as SAURs, PCBP, AtBTs, and CaM-binding WRKYs (Yang and Poovaiah 2000a; Reddy et al. 2002; Du and Poovaiah 2004; Park et al. 2005) and studied only in a limited number of cases with direct experimental evidence, such as GAD, CCaMK, MLO, AtCAT3, DWF1, AtSRs/CaMTAs and CRLK1 (Snedden et al. 1996; Takezawa et al. 1996; Kim et al. 2002a; Yang and Poovaiah 2002b; Du and Poovaiah 2005; Du et al. 2009; Yang et al. 2010). Hence an equally imperative

issue is to study whether and how Ca^{2+}/CaMs or Ca^{2+}/CMLs regulate their target proteins. How a simple messenger, the divalent calcium cation, is encoded into such a spectrum of specific signals and how these encoded Ca^{2+} signals are interpreted into various biochemical/molecular responses which underly various physiological activities with the specificity and accuracy to support precisely coordinated plant life, is the most amazing yet mysterious nature of Ca^{2+}/CaM-mediated signaling. The Ca^{2+} signature hypothesis was introduced to explain the specificity of Ca^{2+}-mediated signaling in which the frequency, duration, intensity, and spatial distribution of Ca^{2+} transients all contain particular meaning(s) and affect the interpretation of the encoded Ca^{2+} signals (Rudd and Franklin-Tong 2001; Scrase-Field and Knight 2003). Stimulus-specific calcium oscillations, a particular signature in guard cell of *Arabidopsis*, was observed to couple with stomatal closure, providing a direct support to the calcium signature hypothesis in plants (Allen et al. 2000). Calmodulin-mediated activation of target proteins is generally accepted to work in a high on/low off mode. Combined with specific regulatory mechanisms of calmodulin-binding proteins, such as CaM trapping and autonomy of kinase activity as well as autodephosphorylation after the initial Ca^{2+}/CaM-mediated activation of CaMKII (Meyer et al. 1992; Hudmon and Schulman 2002), the high on/low off mode could also enable a CaM-regulated protein to produce specific responses to calcium signals with specific signatures (De Koninck and Schulman 1998). However, the molecular mechanisms involving calmodulin which interpret the specific calcium signature, such as frequency in plant cell, are virtually unknown. Although exciting progress has been achieved in our understanding of Ca^{2+}/CaM-mediated signaling in plants, our understanding of this complex network is far from clear. Concerted multidisciplinary approaches involving biophysics, biochemistry, molecular biology, functional genomics, proteomics, metabolics, bioinformatics and imaging techniques should help in our future dissection of the Ca^{2+}/CaM-mediated signaling network in plants.

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The CBL–CIPK Network for Decoding Calcium Signals in Plants

Oliver Batistic, Kyung-Nam Kim, Thomas Kleist, Jörg Kudla, and Sheng Luan

Abstract Like all other eukaryotes, plants utilize calcium as an important secondary messenger for signal transduction. Among plants, calcium signals are particularly important for triggering physiological responses to environmental perturbations or stresses. Calcineurin B-Like proteins (CBLs) are prominent examples of plant calcium-sensor proteins involved in environmental responses. CBLs regulate the activities of a family of SNF1-related kinases, known as CBL-interacting protein kinases (CIPKs), in a calcium-dependent manner. The CBL–CIPK network appears to be present in all land plants and certain green algae, though its functions have only been studied in higher plants. Among higher plants, the network typically consists of about 10 CBLs and 25–30 CIPKs. Through combinatorial interactions among these proteins, the CBL–CIPK network enables integration of multiple signals of the plant's environment and coordinates downstream responses to stresses such as nutrient deprivation or toxic ion exposure. The CBL–CIPK network thus represents a major decoding mechanism for various calcium signatures in response to extracellular cues.

Oliver Batistic, Kyung-Nam Kim, and Thomas Kleist contributed equally to this chapter.

O. Batistic • J. Kudla
Institut für Botanik, Universität Münster, Schlossplatz 4, 48149 Münster, Germany

K.-N. Kim
Department of Molecular Biology, Plant Engineering Research Institute, Sejong University, Seoul 143–747, South Korea

T. Kleist • S. Luan (✉)
Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA
e-mail: sluan@nature.berkeley.edu

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1 Introduction

In order to respond to their environment and coordinate developmental programs, plants have evolved intricate machineries for signal transduction. Most, if not all, plant signaling processes are accompanied by transient, defined changes in cytosolic calcium concentration that are termed calcium signatures (Gilroy and Trewavas 2001; Batistic and Kudla 2010). In order to maintain resolution among the various signals conveyed by calcium signatures, each particular signature needs to be decoded in a highly specified manner. Local and temporal differences in the shape of stimulus-specific signatures may be the primary factors for response specificity. However, additional processes downstream of the formation of calcium signatures provide greater specificity and enable the plant to decide on appropriate molecular responses (Luan et al. 2002). It has become increasingly apparent that plant signal transduction is typically not accomplished via linear pathways. Rather, accumulating evidence indicates that signaling networks predominantly resemble webs or scale-free networks (Hetherington and Woodward 2003; Hetherington and Brownlee 2004; Dodd et al. 2010). Scale-free networks are composed of many interconnected nodes wherein a small number of highly connected nodes, known as hubs, are central to the functionality of the entire network. Scale-free network topology is advantageous for biological signaling networks because it confers robustness and flexibility by permitting alternative pathways for information flow among network nodes (Barabasi and Oltvai 2004). Moreover, such systems are ideally suited for both integrating and discriminating among multiple signals simultaneously.

Collectively, calcium signals are regarded as one of the central hubs of plant signaling networks. Calcium signaling is connected with other signaling systems within the plant cell, and calcium-regulated scale free-like networks are implicated to function as transducers of various calcium signals (Dodd et al. 2010). Calcineurin B-like (CBL) calcium sensor proteins and CBL-interacting protein kinases (CIPKs) constitute an important calcium-based signal transduction network in plants. In the model plant Arabidopsis, the CBL–CIPK network consists of 10 sensor proteins and 26 interacting effector kinases and appears to function as a scale free-like network (Batistic and Kudla 2009; Luan 2009; Weinl and Kudla 2009). In this respect, the CBL–CIPK signaling network is fundamentally different from calcium-dependent protein kinases (CDPKs), which contain calcium sensor and effector kinase domains within single proteins, because it consists of two distinct, freely combinable layers of processing components. This characteristic of the CBL–CIPK network is believed to enable plants to effectively decode and respond to calcium signals stemming from diverse stimuli.

2 Structure, Localization, and Molecular Mechanisms of CBLs and CIPKs

As typical representatives of the calmodulin protein superfamily, CBLs are relatively small proteins (23.5–32 kDa) that consist of four calcium-binding EF hand domains (Nagae et al. 2003; Kolukisaoglu et al. 2004) (Fig. 1). Each EF hand

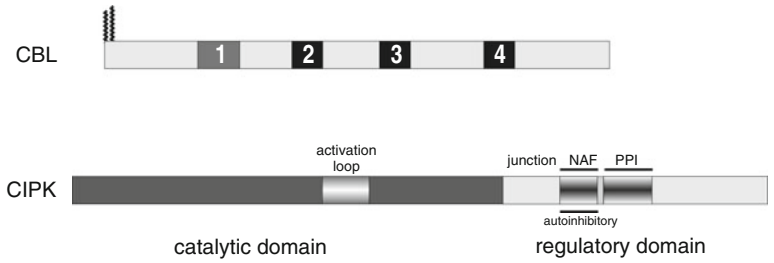

Fig. 1 Domain structure of CBLs and CIPKs. The overall structure of CBLs comprises four EF hands (*boxes with numbers*). While the N- and C-terminal extensions vary in length, the spacing of EF hands in all Arabidopsis CBLs is invariable. In CBLs, the first EF hand represents an unconventional calcium-binding domain encompassing 14 amino acids instead of 12 like in a canonical EF hand (*light gray box*). *Jagged lines* indicate the position of lipid modification sites (N-myristoylation and S-acylation) as found in CBL1 from Arabidopsis. The overall structure of CIPKs consists of an N-terminal kinase domain (*dark gray*) typical of Ser–Thr kinases, which harbors the activation loop (*gray gradient*). The regulatory C-terminal domain (*light gray*) consists of a junction domain which separates the kinase domain from two protein interaction domains (*inverted gray gradient boxes*), the NAF domain, which is responsible for CBL–CIPK interaction, and the adjacent PPI domain responsible for interaction with PP2Cs

domain typically consists of two alpha-helices, which flank a loop of 12–14 amino acids. Within a canonical EF hand, a calcium ion is coordinated by ligands at position 1(X), 3(Y), 5(Z), 7(−Y), 9(−X), and 12(−Z) of the loop, which are typically occupied by negatively charged amino acids (Lewit-Bentley and Rety 2000). Most EF hands of CBLs display substitutions of these negatively charged amino acids by positively charged basic amino acids, especially at the ligand position Y (Kolukisaoglu et al. 2004). Indeed, none of the identified CBL proteins contain four canonical EF hands, in contrast to calmodulins. While CBL1 and CBL9 from Arabidopsis contain two canonical and two noncanonical EF hands, CBL6, CBL7, and CBL10 each contain only a single canonical EF hand. None of the other CBL proteins from Arabidopsis (CBL2, CBL3, CBL4, CBL5, and CBL8) contain any canonical EF hands (Kolukisaoglu et al. 2004). These differences in terms of the number of canonical EF hands suggest that the different proteins exhibit varying affinities for calcium, which likely facilitates the decoding of distinct calcium signals at different local calcium concentrations. As a consequence of these differences in EF hand composition, individual CBL proteins exhibit diverse modes of calcium binding compared to one another or calmodulins. Crystallization studies of CBL2 and CBL4 from Arabidopsis have revealed that, in their unbound states, CBL2 binds two calcium ions and CBL4 binds four calcium ions (Nagae et al. 2003; Sanchez-Barrena et al. 2005). When either calcium sensor is bound to the regulatory domain of an interacting protein kinase, CBL2 binds four calcium ions while CBL4 only binds two (Sanchez-Barrena et al. 2007; Akaboshi et al. 2008). The functional consequences of these different calcium-binding modes are unknown, and it is unclear if these findings indeed reflect the situation *in planta*. Lipid modifications, which are missing in the crystallization studies of proteins that

were purified from *Escherichia coli*, can modify the calcium-binding properties of proteins; therefore the aforementioned experimental results should be interpreted with caution (Ames et al. 1995).

Crystal structure analysis of CBL2 revealed some additional unexpected features that do not appear to be shared by other calcium-binding proteins. Computational analysis of CBL protein sequences revealed that the first EF hands of CBLs contain amino acid substitutions in positions that are critical for binding calcium ions. Instead of the canonical 12 amino acid calcium-binding loop, the first EF hand of CBLs contains a 14 amino acid calcium-binding loop. This initially led to the conclusion that, similar to the first EF hands found in animal and fungal homologs of CBLs that act as calcium sensors and are known as neuronal calcium sensor (NCS) proteins, the first EF hands of CBL proteins do not bind calcium (Kolukisaoglu et al. 2004). However, these atypical first EF hands of CBL proteins have since been shown to indeed bind calcium ions (Nagae et al. 2003). The unexpected calcium-binding ability of these 14 amino acid loops is a result of their unique structures, which enable coordination of calcium ions by the side chain carboxylates of aspartate (position 64 in CBL2 from Arabidopsis) and glutamate (71) at the positions Z and −Z, respectively. Coordinates X (serine 58), Y (isoleucine 62), and −Y (leucine 66) bind the calcium ion via the main chain carbonyl, and the −X coordinates bind calcium ions via a water molecule (Nagae et al. 2003). A further surprise came from the finding that EF hand 4 of CBL2 is also able to bind calcium despite containing a positively charged amino acid at the important calcium-coordinating position Y. Interestingly, the same positively charged residue at position Y in EF hands 2 and 3 seems to be responsible for the inability of these particular EF hands to bind calcium when the protein is not in complex with its interacting kinase. Upon interaction with CIPK14, CBL2 becomes able to bind calcium at all four EF hands, including EF hand 2 and EF hand 3, each of which harbors a positively charged amino acid at the Y position (Akaboshi et al. 2008). CIPK14 appears to promote binding of additional calcium ions to CBL2 by inducing a conformational change in the central region of the calcium sensor upon interaction. These findings may indicate that complex formation and activation of CBL–CIPK complexes could occur at low calcium concentrations and result in prolonged activity of the complex after cytosolic calcium concentration has declined.

Crystal structure studies of the CBL2 and CBL4 proteins from Arabidopsis also revealed that the overall structure of CBLs consists of two globular domains, each of which contains an EF hand pair, separated by a short linker region between the globular domains (Nagae et al. 2003; Sanchez-Barrena et al. 2005). The EF hands are arranged in a linear array along one side of the molecule, which allows interchange of water or calcium molecules from the EF hands. Only the termini of CBL proteins from Arabidopsis vary in length (Kolukisaoglu et al. 2004). Since the central regions of all CBL proteins from Arabidopsis do not vary in their number of amino acid positions, the described globular structure is assumed to be a general feature of all CBL proteins. This structural characteristic of the CBL protein family is shared by the NCS protein family present in animals and fungi

(Strahl et al. 2003). NCS proteins also harbor an N-terminal hydrophobic crevice on the opposite side of their EF hands, which is important for their interaction with effector proteins (Strahl et al. 2003). The crystal structure of CBL2 in complex with the regulatory domain of CIPK14 suggests a similar mode of complex formation. In the CIPK-unbound state of CBL2, the hydrophobic pocket is intramolecularly obstructed by short, C-terminal helices (Nagae et al. 2003). When complexed with the regulatory domain of CIPK14, the C-terminus of CBL2 is shifted away from the hydrophobic pocket. This conformational change permits CBL2 to interact with the hydrophobic NAF domain in the C-terminus of the kinase (Akaboshi et al. 2008). Interestingly, it was shown that the C-terminus of CBL10 can be phosphorylated by its interacting partner CIPK24 at a serine residue at position 237 that is conserved among several other CBL proteins from Arabidopsis, including CBL2 (Dodd et al. 2010). By mimicking phosphorylation of the residue at this position, researchers were able to demonstrate enhanced interaction between CBL10 and CIPK24 both in yeast interaction studies and in vitro. Moreover, a nonphosphorylatable version of CBL10 was found to be unable to complement the salt-sensitive phenotype of *cbl10* mutant plants (Lin et al. 2009). Therefore, it is tempting to speculate that phosphorylation of the C-terminus of certain CBL proteins may hinder the ability of their C-termini to bind to their central hydrophobic crevices and thereby promote CBL–CIPK interaction. Alternatively, electrostatic interactions induced by phosphorylation may lead to a stronger association between CIPKs and the phosphorylated C-termini of certain CBLs.

CIPKs are a monophyletic group of serine–threonine protein kinases that are believed to be the major or exclusive targets of CBLs. CIPKs belong to the third group of Sucrose-non-fermenting (Snf)-related protein kinases (SnRK3s), which are related to Snf1 from yeast and AMP-activated kinase (AMPK) from animals (Hrabak et al. 2003). Protein kinases within this group share a common structural composition (Fig. 1). The N-terminal half of each CIPK contains the kinase domain, which typically contains 11 subdomains that are conserved among serine–threonine kinases (Kolukisaoglu et al. 2004). Remarkably, the kinase activity of CIPKs has an enormous preference for manganese rather than magnesium as a co-factor (Shi et al. 1999). The significance of this rather unusual co-factor preference of CIPKs is currently unknown. The C-terminal half of the CIPK proteins consists of a regulatory domain that includes a junction domain and protein interaction domains (Albrecht et al. 2001). The first, highly conserved interaction domain is termed the "NAF" domain, according to the three amino acids (asparagine, alanine, and phenylalanine) conserved among all known CIPKs and critical for their interaction with CBLs. An isolated NAF domain containing only 24 amino acids was shown to have the ability to interact with CBLs, but it was found to be unable to confer interaction specificity (Albrecht et al. 2001). It is assumed that binding of CBL proteins to the NAF domain of CIPKs releases the kinase domain from the C-terminal regulatory domain, which in a deactivated state acts as a pseudosubstrate and thus serves as an autoinhibitory domain. Thereby the binding of a CBL to a CIPK switches the kinase into an active state (Guo et al. 2001; Gong et al. 2002). It should be noted that interactions between CBLs and CIPKs can occur

independently of calcium, whereas activation of the kinase, particularly towards targets, requires both the CBL protein and calcium ions (Halfter et al. 2000; Guo et al. 2001). This implies that CBL binding to the NAF domain is not the only factor responsible for displacing the autoinhibitory domain from the kinase domain. Conformational changes of the CBL upon calcium binding are required to trigger further structural changes within the kinase that are important for activation towards its substrate(s). CIPKs contain another, less well-conserved protein interaction domain nearer to their C termini. This protein phosphatase interaction (PPI) domain is responsible for selective interactions with class 2C (PP2C) protein phosphatases such as ABI1 and ABI2 (Ohta et al. 2003) or AIP1 (Lee et al. 2007). These observations suggest functional interconnections between calcium-regulated protein kinases and protein phosphatases. The functional consequences of PP2C-CIPK interactions are not fully understood. Nevertheless, it was shown that a CIPK and a PP2C can antagonistically modulate the activity of ion channels when co-expressed in *Xenopus* oocytes (Lee et al. 2007). It is unclear whether the kinase and the phosphatase compete for phosphorylation/dephosphorylation of the channel or if they modulate the activities of each other by dephosphorylation of the kinase and/or phosphorylation of the phosphatase. Structurally, the PPI domain is similar to the kinase-associated domain 1 (KA1) of the KIN2/PAR-1/MARK kinase subfamily (Sanchez-Barrena et al. 2007; Akaboshi et al. 2008). Intriguingly, SnRK2s also interact with PP2Cs to mediate ABA responses in plant cells, and SnRK1, a SNF1 homolog in plants, also contains a similar structural domain that may enable interaction with PP2Cs (Yoshida et al. 2006; Akaboshi et al. 2008; Park et al. 2009).

The activation mechanism of CIPKs implicates a further interconnection of the CBL–CIPK network with other signal transduction systems. Several serine and threonine residues within the activation loop of the kinase domain are modified by *trans*-phosphorylation events brought about by a still unknown kinase. Phosphorylation of the activation loop enables the kinase to efficiently autophosphorylate independently of calcium ions (Guo et al. 2001; Gong et al. 2002). Further work is needed to determine the extent and the molecular mechanisms of cross talk among the CBL–CIPK network and other plant signaling networks.

Although CBLs and CIPKs are conceived as obligate partners in the CBL–CIPK signaling network, it is currently uncertain whether CBLs regulate targets other than CIPKs and whether CIPKs are activated by other calcium sensors. Several reports have indicated that proteins other than CIPKs can also interact with CBLs such as the 5′-methylthioadenosine nucleosidase (MTAN) (Oh et al. 2008) and the phosphatidyl-inositol-4-kinase beta 01 (PI4Kb) protein (Preuss et al. 2006). The latter interaction is reminiscent of the interaction between PIK1 from yeast (the PI4Kb homolog) and frequenin, a calcium-sensor protein related to CBLs (Strahl et al. 2003). However, while MTAN activity is inhibited selectively by calcium and CBL3 (Oh et al. 2008) the functional relevance of the CBL–PI4Kb interaction is not understood. CIPKs may also interact with components of calcium-dependent signaling systems other than CBLs. An investigation of the Arabidopsis interactome using protein microarrays revealed that calmodulins and calmodulin-like (CML) proteins are also able to interact with certain CIPKs (Popescu et al.

2007). It remains unclear whether calmodulins or CMLs can actually activate CIPKs or if they are merely capable of physical interactions. In a further phosphoproteome assay, two MAP kinases were identified which could selectively phosphorylate CIPKs. While MPK7 phosphorylated CIPK24, MPK16 was found to phosphorylate CIPK23 from Arabidopsis (Popescu et al. 2009). Since the position of the phosphorylation sites within the CIPKs that are modified by the MAP kinases is unknown, it is tempting to speculate that these MAP kinases may be responsible for the observed phosphorylation of residues within the activation loops of CIPKs.

CBL proteins can be categorized according to their subcellular localization, and in some cases these categories are consistent with their inferred phylogenetic relationships. CBL1, CBL4, CBL5, CBL8, and CBL9 from Arabidopsis comprise the "plasma membrane-type" group of CBLs. This group arose in part by a recent segmental genome duplication that produced CBL1 and CBL9 in Arabidopsis, which share 89% identity in amino acid sequence. With the exception of CBL8, these proteins contain motifs for N-myristoylation and S-acylation. Together, the two modifications anchor these CBLs to the plasma membrane (Batistic et al. 2008). CBL2, CBL3, CBL6, CBL7, and CBL10 from Arabidopsis comprise the "tonoplast-type" CBL group, although some experimental results suggest that CBL7 is localized within the cytoplasm and the nucleus and may not associate with membranes (Batistic et al. 2010). Like CBL1 and CBL9, CBL2 and CBL3 also arose via segmental duplication, and they share 92% identity in amino acid sequence. Furthermore, CBL7 was produced by a tandem duplication of the CBL3 gene, but it has undergone dramatic changes in its amino acid sequence since. Notably, CBL7 does not contain the extended N-terminal domain shared by most CBLs in this group. Additionally, the first EF hand of CBL7 differs from the first EF hand of all other CBLs in that it lacks the serine at position X, which is important for calcium coordination in this unconventional calcium-binding loop. The third EF hand also contains a substitution at the crucial −Z position, which is changed from aspartate to glycine. Overall, CBL7 exhibits only 60% amino acid sequence similarity with CBL3 (Kolukisaoglu et al. 2004).

The N-terminal domains of CBLs determine their subcellular localization. CBL1, CBL4, CBL5, and CBL9 from Arabidopsis each contain a classical N-myristoylation site, and it was shown that these proteins are indeed N-myristoylated (Batistic et al. 2008). CBL1, CBL4, and CBL9 also contain one cysteine residue and CBL5 contains two cysteine residues adjacent to the myristoylated glycine, which could be further targets for S-acylation. S-acylation indeed occurs at the cysteine-3 residue of CBL1, and it is highly likely that the other aforementioned CBLs are also S-acylated (Batistic et al. 2008). This modification is crucial for the localization as well as the function of CBL1 (Batistic et al. 2008). While the myristoylated CBL1 protein is targeted to the ER, subsequent S-acylation results in final targeting of the protein to the plasma membrane. Importantly, only a wild-type cDNA of CBL1 can complement the salt-sensitive phenotype of a *cbl1* mutant; altered cDNA sequences that encode nonmyristoylatable or nonpalmitoylatable versions of CBL1 were unable to complement this mutant (Batistic et al., 2008). CBL4, CBL5, and CBL9 are also targeted to the plasma membrane and are believed

to use similar targeting mechanisms based on similarity in their N-terminal amino acid sequences (D'Angelo et al. 2006; Cheong et al. 2007; Batistic et al. 2010). N-terminal domains of these proteins have been shown to be sufficient to target GFP to the plasma membrane. CBL8 presents a special case with regard to its subcellular targeting. Phylogenetic analysis revealed that CBL8 belongs to the plasma membrane group, and CBL8 was shown to indeed bind to the plasma membrane to some extent (Kolukisaoglu et al. 2004; Batistic et al. 2010). However, in contrast to the other CBLs from the same group, the CBL8 protein lacks the classical myristoylation site. Unlike the full-length protein, the N-terminal domain of CBL8 containing amino acids 1–16 is fully sufficient to target GFP efficiently to the plasma membrane. The lack of a myristoylation site in CBL8 suggests that in contrast to the other plasma membrane type proteins, CBL8 bypasses the primary binding to the endoplasmic reticulum mediated by the N-myristoyl group and may be targeted directly to the plasma membrane. Interestingly, the efficiency of plasma membrane targeting of CBL8 appears to be enhanced by interaction with CIPKs, since BiFC analysis of CBL8 in complex with CIPK14 revealed exclusive plasma membrane localization of the CBL8/CIPK14 complexes. Together with the exclusive plasma membrane localization mediated by the isolated CBL8 N-terminus, this finding may indicate that the conformation of the full-length CBL8 protein when it is not in complex with a CIPK may interfere with plasma membrane targeting by its N terminus (Batistic et al. 2010).

CBL2, CBL3, CBL7, and CBL10 from Arabidopsis comprise a second category of CBLs with regard to their subcellular localization. With the exception of CBL7, all proteins harbor extended N-terminal domains and are known to localize to the vacuolar membrane (Batistic et al. 2010). CBL10 is also targeted to an endosomal compartment, which is unique among studied CBLs (Kim et al. 2007; Batistic et al. 2010). Like other CBLs, the N-terminal domain mediates the specific targeting of these proteins. A short fragment of the N-terminal domain of CBL3 (amino acids 1–22) was shown to be sufficient for efficient targeting of GFP to the tonoplast. The N terminus of CBL10 (amino acids 1–40) contains a predicted transmembrane domain, and it is also sufficient to target GFP to the tonoplast and endosomal vesicles. However, the targeting mechanisms of CBL3 and CBL10 appear to be different, as CBL3 lacks a predicted transmembrane domain, which was shown to be necessary for CBL10 to bind to membranes (Batistic et al. 2010). It has been suggested that CBL10 may undergo alternative splicing to produce different N-terminal sequences (Kolukisaoglu et al. 2004). Since the putative differences would occur at the extreme N terminus, the transmembrane domain is likely unaffected by alternative splicing, which suggests that all potential CBL10 isoforms should be targeted to endosomal vesicles and the tonoplast.

All CIPK proteins analyzed so far appear to be soluble proteins that are localized to the cytosol and the nucleus when fused to GFP, and CBL N-terminal factors are believed to determine the subcellular localization of CBL–CIPK complexes (Kolukisaoglu et al. 2004; D'Angelo et al. 2006; Cheong et al. 2007; Batistic et al. 2010). The lack of recognizable targeting signals such as N-terminal myristoylation sites or prenylation sites at the C-terminal end of CIPKs further

suggests that CBLs are the sole factors that determine targeting of their respective kinases to various membranes. BiFC analyses elegantly showed that CIPKs can form alternative complexes with different CBLs at either the plasma membrane or the tonoplast (Kim et al. 2007; Batistic et al. 2008; Waadt et al. 2008). Importantly, multicolor BiFC analysis revealed that this alternative complex formation can occur simultaneously within a single cell, as shown for CBL1–CIPK1 and CBL9–CIPK1 at the plasma membrane or for CBL1–CIPK24 and CBL10–CIPK24 at the plasma membrane and the vacuolar membrane, respectively. This observation confirms the working model that CBLs and CIPKs form an intricate, web-like network within the cell and that the identity of the CBL protein determines the cellular localization of CBL–CIPK complexes (Waadt et al. 2008).

Aside from the differential localization of CBL proteins, preferential complex formation of particular CBLs with defined subsets of CIPKs and vice versa appears to confer further specificity within the CBL–CIPK network, and this network characteristic is reflective of scale-free network architecture. Indeed, interaction analyses revealed that some CBLs (e.g. Arabidopsis CBL2) act as network hubs since they appear to interact with many CIPKs, whereas other CBLs appear to only interact with a restricted number of CBLs (Albrecht et al. 2001). The kinase domain of CIPKs may play a partial role in determining the specificity of CBL–CIPK interactions (Kim et al. 2000). In yeast two-hybrid studies, it was observed that Arabidopsis CBL4 interacted with the C-terminal regulatory domains of both CIPK5 and CIPK6. In contrast, CBL4 only interacted with CIPK6 when full-length CIPKs were used in the analysis. Intriguingly, CBL4 was unable to interact with a chimeric protein containing the kinase domain of CIPK5 fused to the regulatory domain of CIPK6 (Kim et al. 2000). Considering that other yeast two-hybrid analyses revealed an interaction between isolated N-terminal and C-terminal domains of CIPK24/SOS2 (Guo et al. 2001), the inability of the chimeric CIPK5/CIPK6 protein to interact with CBL4 may stem from improper folding of the chimeric protein due to incompatibility between the N-terminal and C-terminal domains.

Another aspect that confers network specificity is the differential expression of network components in response to external stimuli. Differences in expression patterns have been observed even among closely related CBL proteins, for example CBL1 and CBL9 in Arabidopsis. While CBL1 gene expression is much more strongly induced by cold treatment than CBL9, CBL9 gene expression is much more strongly induced by ABA than CBL1 (Kilian et al. 2007). CBLs and CIPKs can be also differentially expressed in various plant tissues. For example, CBL4 is mainly expressed in roots, whereas CBL10 is mainly expressed in leaves. Both proteins interact with CIPK24, which is expressed in both plant tissues, to regulate ion homeostasis under salt stress (Liu et al. 2000). Furthermore, the two complexes act at different compartments within the cell. While CBL4–CIPK24 complexes are localized at the plasma membrane to regulate the sodium–proton exchanger SOS1 (Qiu et al. 2002), CBL10–CIPK24 complexes form mainly at the vacuole (Kim et al. 2007). Vacuolar localization of the CBL10–CIPK24 complex may reflect the need of leaves to sequester toxic sodium ions in the vacuole, since extrusion of

sodium to the apoplast could interfere with apoplastic calcium that is bound to the cell wall and cell membrane to provide structural stability (White and Broadley 2003; Batistic and Kudla 2010).

Another important mechanistic aspect of network regulation is the differential activation of CBL–CIPK complexes towards target proteins. Studies of Arabidopsis CIPK1 revealed that it is differentially regulated by the closely related proteins CBL1 and CBL9 (D'Angelo et al. 2006). While interaction of CBL1–CIPK1 is important for regulating downstream processes during salt stress, interaction of CBL9–CIPK1 is crucial for responses to the stress-hormone ABA (Albrecht et al. 2003; Pandey et al. 2004; D'Angelo et al. 2006). However, CBL1 and CBL9 have also been shown to have overlapping functions, as they function together in the activation of CIPK23 to trigger phosphorylation of the target protein AKT1 (Li et al. 2006; Xu et al. 2006). CIPK23 also interacts with CBL5 and CBL8, but these sensors are unable to regulate CIPK23 activity towards its target AKT1. On the other hand, CIPK24 can also interact with CBL1 and CBL9, yet CIPK24 is unable to activate AKT1 (Geiger et al. 2009). This indicates that interaction of specific CBLs with CIPKs definitely modulates the target specificity of CIPKs. This hypothesis is further supported by complementation tests of CBL mutants. For example, only a cDNA encoding CBL1 was able to complement a *cbl1* mutant line. A cDNA encoding CBL2, which interacts with the exact same set of CIPKs as CBL1 but is localized to the tonoplast, was unable to complement the *cbl1* salt-sensitive phenotype even when the CBL2 protein was altered to target it to the plasma membrane (Batistic et al. 2008). The aforementioned observations indicate that there are factors that confer specificity to CIPK kinase activity that cannot be determined through assays that simply detect physical interactions with CBLs. The molecular and structural nature of these factors has yet to be characterized.

3 Evolution and Functional Diversification of CBLs and CIPKs

The increasing number of available genome and transcriptome sequences in recent years has facilitated analysis of the evolution of the CBL–CIPK signaling system. Single CBL and CIPK genes have been identified in green algae species of the genera *Ostreococcus* and *Chlorella* (Batistic and Kudla 2009; Weinl and Kudla 2009). These findings establish that the CBL–CIPK system evolved prior to the split between green algae and the lineage leading to modern plants and point to a role for the CBL–CIPK signaling system in single-celled organisms. Remarkably, CBL and CIPK proteins appear to be absent in the well-studied green algae *Volvox carteri* and *Chlamydomonas reinhardtii*. Computational analyses have revealed that these algal genera appear to follow animal paradigms in other aspects of their calcium signaling machinery. For example, *Chlamydomonas* contains Ca^{2+}-releasing IP3 receptors that are found among animals but appear to be absent from land plants (Wheeler and Brownlee 2008). This situation suggests that certain chlorophyte green algae, represented by *Chlamydomonas* and *Volvox*, may rely on a calcium-signaling

system that is distinct from other plants. This distinction may reflect their need for more rapid forms of calcium signal transduction due to their motility, which sharply contrasts with the sessile lifestyle of land plants and more closely related charophyte green algae (Verret et al. 2010).

In contrast to studied green algal species, which each appear to contain a single CBL and CIPK, the moss *Physcomitrella patens* contains four CBLs and seven CIPKs; and the fern ally *Selaginella moellendorffii* contains four CBLs and five CIPKs genes (Batistic and Kudla 2009; Weinl and Kudla 2009; Batistic et al. 2010). These observations suggest that the complexity of the CBL–CIPK system evolved concurrently with the increasing morphological, physiological, and developmental sophistication of land plants that enabled their colonization of diverse and labile environments. This expansion in network size and complexity is paralleled by diversification of the subcellular localization of CBL proteins. The single CBLs from *Ostreococcus lucimarinus* and *Chlorella* are similar to plasma membrane-type CBLs from land plants. These CBLs harbor classical myristoylation sites with additional potential for palmitoylation at adjacent cysteine residues, which may be the mechanism for targeting to the plasma membrane. In the early diverging land plants *Physcomitrella* and *Selaginella*, the CBL family is expanded relative to green algae. In addition to plasma membrane-type CBLs, these basal land plants appear to contain tonoplast-type CBLs that cluster together with Arabidopsis CBL2 and CBL3 in phylogenetic analyses (Batistic et al. 2010). Despite its phylogenetic placement with tonoplast-type CBLs from Arabidopsis, one of the two apparently tonoplast-type *Physcomitrella* CBLs, CBL2, contains the classical myristoylation and palmitoylation sites found among plasma membrane-type proteins, and it lacks the N-terminal extension that is characteristic of CBLs within this group. These observations suggest that CBL2 from *Physcomitrella* may actually be targeted to the plasma membrane rather than the vacuole. In addition to its role as a critical intracellular storage compartment, the vacuole is known to play a critical role in calcium signal transduction. CBL–CIPK pairs may be involved in the regulation of both of these vacuolar functions, and this may be the reason that tonoplast-localized CBLs appear to have evolved very early in land plant evolution.

In higher plants, the CBL–CIPK system further expanded into a large, complex network of proteins. Bioinformatic analyses of both protein families have identified a complement of 10 CBLs and 26 CIPKs in the Arabidopsis genome and 10 CBLs and 30 CIPKs in the rice genome (Albrecht et al. 2001; Kolukisaoglu et al. 2004; Weinl and Kudla 2009). Recent studies identified ten genes encoding CBL proteins and 25 CIPKs in the fully sequenced genome of *Populus trichocarpa* (Yu et al. 2007; Zhang et al. 2008; Weinl and Kudla 2009). Similar investigations of other fully sequenced plant genomes have indicated that the dicot *Vitis vinifera* contains 8 CBLs and 21 CIPKs and the monocot *Sorghum bicolor* contains 6 CBLs yet 32 CIPKs (Weinl and Kudla 2009). The expansion of the CBL family within flowering plants produced a further subset of CBLs, represented by Arabidopsis CBL10, that is lacking in *Selaginella* and *Physcomitrella* (Weinl and Kudla 2009). The proteins within this group are unique in regard to their N-termini, which harbor predicted transmembrane domains not found among other CBLs. The similarity among

CBL10-type proteins from different species may reflect similar subcellular distribution and functionality, as Arabidopsis CBL10 displays a unique localization pattern to both the tonoplast and endosomal vesicles (Kim et al. 2007; Batistic et al. 2010). This may constitute another example of functional diversification during the evolution of CBLs through localization to new subcellular destinations.

Phylogenetic analyses have indicated that all CIPKs from *P. patens* and *S. moellendorffii* array together with CIPK23 and CIPK24 from Arabidopsis (Weinl and Kudla 2009). AtCIPK23 and AtCIPK24 have been shown to be critical regulators of plant K^+ and Na^+ homeostasis, respectively (Zhu 2003; Xu et al. 2006). This result may reflect an original function of the ancient CBL–CIPK system in regulating the transport and distribution of these ions in primordial land plants and points to a general, important function of plant CBL–CIPK systems in regulating ion homeostasis. Moreover, all CIPKs from *Physcomitrella* and *Selaginella* harbor introns while all higher plant CIPK families contain a clade that lacks introns in their coding sequence (Kolukisaoglu et al. 2004). This suggests that these intron-free CIPKs evolved after the split of the lycophyte *Selaginella* from the lineage that gave rise to higher land plants. In Arabidopsis as well as in rice, this subgroup represents the larger fraction of CIPK genes in each genome, which could reflect the gain of novel functions besides regulating ion homeostasis in these higher plants (Kolukisaoglu et al. 2004; Weinl and Kudla 2009).

Surprisingly, putative CBLs and CIPKs were recently identified in protozoan human pathogens such as *Trichomonas vaginalis* and *Naegleria gruberi*. This discovery prompts questions about the function(s) of these Ca^{2+}-decoding components in nonplant species (Batistic and Kudla 2009). Remarkably, these calcium sensor proteins seem to harbor myristoylation motifs but lack the typically co-occurrent palmitoylation sites at their N-termini. Even among these protozoan species, the calcium-binding loop of the first EF hand of the CBL proteins is composed of 14 amino acids, pointing to a very early structural diversification of this class of calcium-binding proteins and to an important functional consequence of the unusual structure of the first EF hand (Fig. 2). The CBL-interaction module or NAF domain is also found in CIPKs from *Naegleria* and *Trichomonas*, and the three representative amino acids N, A, and F of the "NAF domain" appear to be invariant. The presence of the PPI domain is less strictly conserved. Considering the

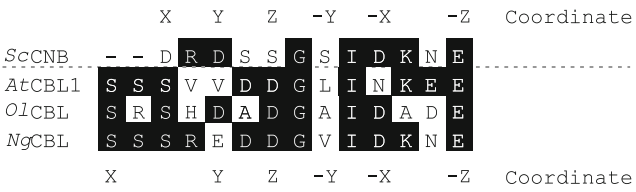

Fig. 2 Comparison of the first EF hand. Comparison of the first EF hands of yeast calcineurin B (*Sc*CNB), Arabidopsis CBL1 (*At*CBL1), *Ostreococcus lucimarinus* CBL (*Ol*CBL) and *Naegleria gruberi* CBL (*Ng*CBL). The position of amino acids which coordinate the calcium ion is indicated *above* (for *Sc*CNB) and *below* (for CBLs) the amino acid alignment. *Black boxing* illustrates identical amino acids

absence of CBL–CIPK proteins in the human hosts of these protozoans, further advancements of our understanding of the regulation of plant calcium-sensor proteins may facilitate the identification of therapeutic inhibitors specifically affecting the "plant-like" proteins of these protozoan species. More broadly, our understanding of the functions of the CBL–CIPK network in organisms other than higher plants remains largely speculative at this time and warrants investigation.

4 Principal Functions of CBLs and CIPKs

Genetic screens aiming to identify critical components of plant salt tolerance provided the first insights into the physiological function of CBLs and CIPKs in regulating plant ion homeostasis. The calcineurin B-like protein SOS3 (AtCBL4) and the CIPK protein SOS2 (AtCIPK24) appear to be part of a calcium-regulated signaling pathway that specifically mediates salt stress adaptation by regulating the plasma membrane Na^+/H^+ antiporter SOS1 (Liu and Zhu 1998; Halfter et al. 2000; Qiu et al. 2002). Studies have demonstrated that the CBL4/SOS3–CIPK24/SOS2 complex phosphorylates and activates the downstream component SOS1, a Na^+/H^+ antiporter (Shi et al. 2000). The activated SOS1 protein then functions to expel excess Na^+ in plant cells, thereby conferring salt tolerance (Qiu et al. 2002).

Recent studies revealed that mutation of Arabidopsis CBL10 also renders plants salt sensitive and that, like CBL4, CBL10 is able to interact with CIPK24 and appears to activate it (Kim et al. 2007; Quan et al. 2007). In vivo analyses revealed that the CBL10–CIPK24 complex localizes to the tonoplast, which supports a functional model wherein alternative complex formation of CIPK24 with either CBL4 or CBL10 creates a dual-functioning kinase that can be targeted to multiple subcellular locations. While CBL4–CIPK24 complexes mediate Na^+ extrusion via the regulation of the Na^+/H^+ antiporter SOS1 at the plasma membrane, the CBL10–CIPK24 complex likely results in Na^+ sequestration into the vacuole by regulating a currently unidentified Na^+ channel or transporter (Kim et al. 2007; Weinl and Kudla 2009). This hypothesis is supported by the observation that *cbl10* mutant plants accumulated much less Na^+ than wild-type plants under both normal and high salt conditions (Kim et al. 2007). The plasma membrane-localized CBL4 is mainly expressed in roots, whereas the tonoplast-localized CBL10 is expressed predominantly in shoots and leaves (Kim et al. 2007). This could reflect a strategy in which plants respond to salt stress primarily through exclusion of Na^+ in their roots, where toxic sodium ions can be returned to the soil, and sequestration of Na^+ in shoots, where sodium ions cannot be entirely expelled from the plant. However, the situation may actually be more complex. CBL1 also appears to be involved in response to salt stresses, as evidenced by decreased salt tolerance in *cbl1* mutant lines (Albrecht et al. 2003; Cheong et al. 2003). Like CBL4, CBL1 is localized to the plasma membrane. Unlike CBL4, CBL1 is expressed both in roots and shoots; therefore CBL1 may also interact with CIPK24 and stimulate Na^+ extrusion in shoots and roots.

In addition to the regulation of Na^+ transporters, recent studies have suggested that CBL–CIPK24 complexes at the tonoplast activate and regulate the vacuolar Ca^{2+}/H^+ antiporter CAX1 independently of CBL4 (Cheng et al. 2004). The CBL involved in the regulation of CAX1 has not yet been identified. Cytosolic levels of Ca^{2+} and Na^+ ions are interrelated not only in that calcium signaling has been shown to play a role in response to high salt conditions, but Ca^{+2} ions also directly participate in the inhibition of the entry of Na^+ ions into the cell (Cheng et al. 2004). CIPK24 was also reported to interact with subunits of the vacuolar ATPase, which suggests that CIPK24 is involved in energizing the vacuolar membrane (Batelli et al. 2007). The resulting proton concentration gradient is required for the activity of tonoplast-localized Na^+/H^+ antiporters such as the NHX transporter family, therefore CIPK24 appears to coordinate several critical cellular responses to high salinity (Batelli et al. 2007).

Recent experimental results suggest that other CIPKs may also be involved in plant responses to salt stress. An Arabidopsis mutant lacking CIPK6 activity was reported to be more sensitive to salt stress compared to wild-type plants, suggesting that CIPK6 plays a role in salt tolerance (Tripathi et al. 2009). Interestingly, CIPK6 has also been shown to physically interact with CBL4 in yeast two-hybrid assays (Kim et al. 2000). Further work will be required to clarify if CIPK6 is indeed involved in salt tolerance and reveal the underlying molecular mechanisms. Moreover, it remains distinctly possible that additional CBLs and CIPKs also function in responses to salt stresses and have yet to be identified.

Aside from mediating responses to adversely high levels of sodium, the CBL–CIPK network is likewise involved in maintaining homeostasis of other important ions in the plant cell, including vital mineral nutrients. In the plant cell, potassium is the most abundant cation, and it serves numerous important functions. Efficient uptake of potassium, especially under high sodium conditions, is critical for plants (Luan et al. 2009). A high-affinity K^+ uptake mechanism is induced within six hours of potassium deprivation (Shin and Schachtman 2004). The underlying system that triggers this induction is dependent on ethylene production, which stimulates a subsequent oxidative burst by activating NADPH oxidases and cell wall-bound peroxidases (Shin and Schachtman 2004; Shin et al. 2005; Jung et al. 2009; Kim et al. 2010). Reactive oxygen species can induce calcium transients (Pei et al. 2000; Foreman et al. 2003), which suggests that a calcium-based signaling system may mediate responses to low potassium conditions (Fig. 3). Indeed, a genetic screen uncovered a role for CBL–CIPK network members in regulating K^+ homeostasis and provided the first molecular insights into the mechanisms by which plant ion channels may be regulated by phosphorylation (Xu et al. 2006). Arabidopsis CIPK23 has been identified as a crucial regulator of high-affinity potassium uptake. CIPK23 is targeted to the plasma membrane and activated by the two closely related calcium sensors, CBL1 and CBL9 (Li et al. 2006; Xu et al. 2006). CBL1–CIPK23 and CBL9–CIPK23 complexes regulate the activity of the shaker-like potassium channel AKT1, which is mainly expressed in root cells where it is important for high-affinity potassium uptake and overall plant nutrition (Lagarde et al. 1996; Hirsch et al. 1998; Li et al. 2006; Xu et al. 2006).

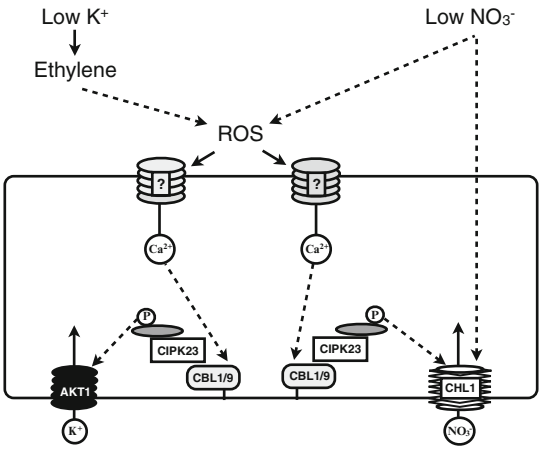

Fig. 3 Regulation of potassium and nitrate uptake by CBL1–CBL9 and CIPK23. Potassium deprivation ("Low K^+" signal) results in activation of a high-affinity uptake mechanism. The signaling system is based on the production of ethylene and subsequent generation of an oxidative burst (ROS). This oxidative burst could mediate the influx of calcium from the apoplast, which is sensed by the plasma membrane-bound sensors CBL1 and CBL9. Calcium binding to the CBLs thereby results in activation of the kinase CIPK23 which subsequently phosphorylates and activates the high-affinity potassium channel AKT1. Similarly, the "Low NO_3^-" signal upregulates the activity of the nitrate transporter CHL1 to stimulate high-affinity nitrate uptake. Similarly to the "Low K^+" signal, nitrate deprivation may result in an oxidative burst, which enables calcium influx and activation of CBL1–CBL9 and CIPK23. Subsequently, CIPK23 phosphorylates CHL1 at threonine 101 which brings about the switch from a low-affinity to a high-affinity nitrate transporter. In addition, at low concentrations nitrate binds solely to the high-affinity site of the CHL1 transporter at the apoplastic face, which appears to be important to modulate the conformation of the transporter at the cytoplasmic face. This conformational change is a prerequisite for phosphorylation of threonine 101 by CIPK23. It should be noted that it is still uncertain whether the oxidative burst indeed mediates calcium influx in response to low potassium or low nitrate

Interestingly, CIPK23 seems to exclusively regulate AKT1; it does not appear to regulate other K^+ transporters from Arabidopsis (Hedrich and Kudla 2006; Lee et al. 2007; Geiger et al. 2009). Patch-clamp studies have revealed that in *cipk23* and *cbl1–cbl9* double mutants, AKT1 activity is significantly reduced (Li et al. 2006; Xu et al. 2006). In contrast, *cbl1* or *cbl9* single mutants do not display reduced AKT1 activity, implicating a functional synergy between CBL1 and CBL9 (Xu et al. 2006; Cheong et al. 2007). Furthermore, interaction analyses in yeast and electrophysiological studies indicated that the 2C-type protein phosphatase AIP1 is a negative regulator of AKT1 that counteracts activation by CIPK23 (Lee et al. 2007; Luan 2009). Although it was shown that kinase activity is important for activation of AKT1, it must be considered that AIP1 can interact with both CIPK23 and AKT1. Due to this intriguing observation, it will be most interesting to distinguish if the activation/deactivation switch of AKT1 is brought about by phosphorylation/dephosphorylation or, alternatively, if it results from competitive binding of either the phosphatase or the kinase to the ankyrin

interaction module of the potassium channel. Aside from the regulation of K^+ uptake in roots, the CBL1/CBL9–CIPK23 module also appears to be involved in stomatal regulation under dehydrating conditions. Both *cbl1/cbl9* double mutants and *cipk23* single mutants exhibit a drought-resistant phenotype, and these mutants are impaired in their regulation of stomatal aperture in response to the hormone ABA (Cheong et al. 2007). This may indicate that the CBL1/CBL9–CIPK23 complex not only mediates the uptake of potassium in roots but also facilitates accurate and appropriate distribution of K^+ throughout green tissues via the transpiration stream.

An exciting novel twist in our understanding of CIPK23 function came from the recent report that this kinase also phosphorylates the nitrate transporter CHL1 (also called NRT1.1). Taking advantage of a novel CHL1 mutant allele (chl1–9), Yi-Fang Tsay and colleagues provided compelling evidence that this mutation impairs the nitrate uptake function of CHL1 without affecting the signaling response to nitrate as analyzed by the transcriptional response of the NRT2.1 gene (Ho et al. 2009). Importantly, biochemical and reverse genetics analyses demonstrated that CIPK23 controls the switch between low- and high-affinity nitrate transport modes by phosphorylating residue Thr101 of CHL1 (Fig. 3). Specifically at low external nitrate concentrations, CIPK23-mediated phosphorylation results in low-level nitrate signaling (Ho et al. 2009). Moreover, Ho et al. (2009) reported that CIPK23 is independently involved in potassium and nitrate responses, indicating a lack of cross talk between the two ions. Considering the critical involvement of the same calcium-sensor proteins (CBL1 and CBL9) and CIPK23-dependent phosphorylation in both processes, this puzzling observation underscores the urgent need for further investigation of the primary ion-sensing mechanism(s) that confers this remarkable specificity in plant signaling. Nevertheless, nitrogen-deficient plants show enhanced ROS production, and the expression of several NADPH oxidases and peroxidases is induced by nitrogen-deficient conditions (Kim et al. 2010). This could implicate that, similar to potassium deprivation, ROS production results in an increase of cytosolic calcium ions and subsequent activation of CBL–CIPK complexes. Arabidopsis CIPK8 also appears to be involved in nitrate responses, as its expression is induced by nitrate and disruption of the CIPK8 gene reduced transcriptional upregulation of nitrate responsive genes. This may reflect a function for CIPK8 as well as CIPK23 in nitrate responses (Hu et al. 2009). Whereas CIPK23 inhibits the high-affinity response to nitrate, CIPK8 mediates the low-affinity response, which suggests that CHL1 is not modulated by CIPK8 (Ho et al. 2009).

More recent work performed in rice has further expanded the known physiological functions of CIPKs to include the integration of nutrient availability sensing with the regulation of plant metabolism (Lee et al. 2009). The results from this study indicate that the protein kinase CIPK15 plays a key role in O_2-deficiency tolerance in rice and is required for growth of rice under flooded conditions. Moreover, CIPK15 regulates the plant global energy and stress sensor SnRK1A, thereby integrating responses to O_2 deficiency with sugar signaling and enabling rice to grow underneath floodwater. The latter finding may point to a general role of

the CBL–CIPK system in fine tuning plant metabolism in response to adverse environmental conditions.

In addition to conveying signals of nutrient deprivation and toxic ion exposure, the CBL–CIPK network is also involved in responses to other abiotic stresses such as osmotic stress, which is not surprising given the importance of potassium in plant osmoregulation and the osmotic stress component of sodium toxicity. Loss-of-function *cbl1*, *cbl9*, and *cipk1* mutants were found to be more sensitive to osmotic stress than the wild type (Pandey et al. 2004; D'Angelo et al. 2006). Several lines of evidence demonstrated that CIPK1 interacts alternatively with either CBL1 or its closest isoform CBL9 at the plasma membrane (Shi et al. 1999; Kim et al. 2000; Albrecht et al. 2001; D'Angelo et al. 2006). These data together suggest that both CBL1 and CBL9 can target CIPK1 to form two distinct complexes, CBL1–CIPK1 and CBL9–CIPK1, and both complexes are required to mediate osmotic stress responses. Interestingly, however, each complex appears to function in a different fashion because the Arabidopsis null mutants responded differently to ABA. Although both *cbl9* and *cipk1* mutants exhibited enhanced sensitivity to exogenous ABA compared to the wild-type plants, the *cbl1* mutant did not show significant changes in ABA responsiveness (Pandey et al. 2004; D'Angelo et al. 2006). Therefore, it appears that CIPK1 acts as a convergence point for ABA-independent and ABA-dependent osmotic stress responses by forming complexes with CBL1 and CBL9, respectively.

As indicated by previous examples, calcium signaling is an important component of ABA signal transduction. ABA plays a critical role in plant responses to abiotic stresses such as drought and high salt (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2006). A specific calcium signature is known to be responsible for mediating early ABA signaling (Leung and Giraudat 1998; Allen et al. 2000; Allen et al. 2001), implicating involvement of calcium sensors in the ABA signaling pathway. It appears that signaling steps downstream of calcium sensing include protein phosphorylation and dephosphorylation. Disruption of the ABI1 or ABI2 genes encoding homologous 2C-type protein phosphatases in Arabidopsis confers an ABA-insensitive phenotype during both seed germination and seedling growth (Leung et al. 1994; Meyer et al. 1994; Leung et al. 1997; Koornneef et al. 1998). Transient expression analyses in maize protoplasts demonstrated that Ca^{2+}-dependent protein kinases (CDPK1 and CDPK1a) are involved in regulating ABA-inducible gene expression (Sheen 1996). In addition, genetic analysis of Arabidopsis mutants showed that two SNF1-related protein kinases, SnRK2.2 and SnRK2.3, are required for ABA signaling (Fujii et al. 2007).

Judging from the fact that calcium and reversible protein phosphorylation are important components for ABA signal transduction pathways, it is not difficult to speculate that some members of the CBL and CIPK families may take part in ABA signaling. Indeed, several lines of evidence supporting this speculation have been reported. Disruption of the CIPK3 gene in Arabidopsis rendered plants hypersensitive to exogenous ABA during seed germination and resulted in significantly lower expression levels of ABA-induced genes such as RD22 and RD29B (Kim et al. 2003). Interestingly, cold-induced expression of RD29A and Kin1/Kin2 genes was

also significantly altered in the *cipk3* mutant, and this effect is largely independent of endogenous ABA production (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). CBL9 forms a specific complex with CIPK3 during ABA-regulated seed germination (Pandey et al. 2008), and loss of Arabidopsis CBL9 gene function also caused plants to become hypersensitive to ABA in early developmental stages such as seed germination and postgermination seedling growth. The *cbl9* mutant seedlings also accumulated much higher levels of ABA than wild-type or complemented plants under osmotic stress imposed by mannitol and salt. Furthermore, the mutant plants displayed elevated expression of genes involved in ABA signaling, such as ABA-insensitive 4 and 5, under osmotic stress conditions or exogenous application of ABA (Pandey et al. 2004). Therefore, it appears that CBL9 is involved in regulation of ABA biosynthesis and sensitivity in Arabidopsis. However, mutation of CBL1, which is very closely related to CBL9, did not affect ABA responsiveness, although it impaired plant responses to drought and salt stresses and affected the expression of cold-regulated genes (Albrecht et al. 2003; Cheong et al. 2003). Silencing of the Arabidopsis CIPK15 gene (designated PKS3 in the paper) by an RNA interference technique rendered plants hypersensitive to ABA in regard to seed germination, seedling growth, stomatal closing, and gene expression (Guo et al. 2002). These observations suggest that CIPK15 may be a global negative regulator of ABA responses. Because yeast two-hybrid assays showed that CIPK15 associates with a group of CBL family members including CBL1, 2, 3, 5, 8, and 9 (K.N. Kim, unpublished data), it seems likely that CBL9 may also form a complex with CIPK15 to mediate ABA responses, though further genetic analysis is required to demonstrate this notion. The aforementioned discoveries provide further indications that CBL–CIPK network members act as cross-talk nodes between ABA-dependent and ABA-independent pathways in stress responses.

CBLs and CIPKs appear to be involved in signaling other distinct environmental conditions such as pH. Arabidopsis mutant plants lacking the functional protein kinase CIPK11 (designated PKS5 in the paper) were shown to be more tolerant to high external pH than the wild type, and this phenotype results from an increased rate of H^+ secretion to the extracellular space (Fuglsang et al. 2007). Further biochemical studies have revealed that CIPK11 negatively regulates the plasma membrane proton pump (PM H^+-ATPase 2; AHA2) by phosphorylating the Ser-931 residue in the C-terminal regulatory domain of AHA2. The phosphorylation of AHA2 prevented interaction with an activating 14-3-3 protein (Fuglsang et al. 2007). Based on interaction studies using yeast two-hybrid and co-immunoprecipitation assays, it was originally proposed that CBL2 associates with and activates CIPK11 in the presence of cytosolic calcium signals elicited by external high pH conditions (Fuglsang et al. 2007). However, CBL2 was recently demonstrated to exclusively localize to the tonoplast (Batistic et al. 2008), which rules out the involvement of the CBL2–CIPK11 complex in regulating the plasma membrane proton pump AHA2. Therefore, the actual CBL member(s) responsible for calcium dependent modulation of the AHA2 activity *in planta* remains to be discovered.

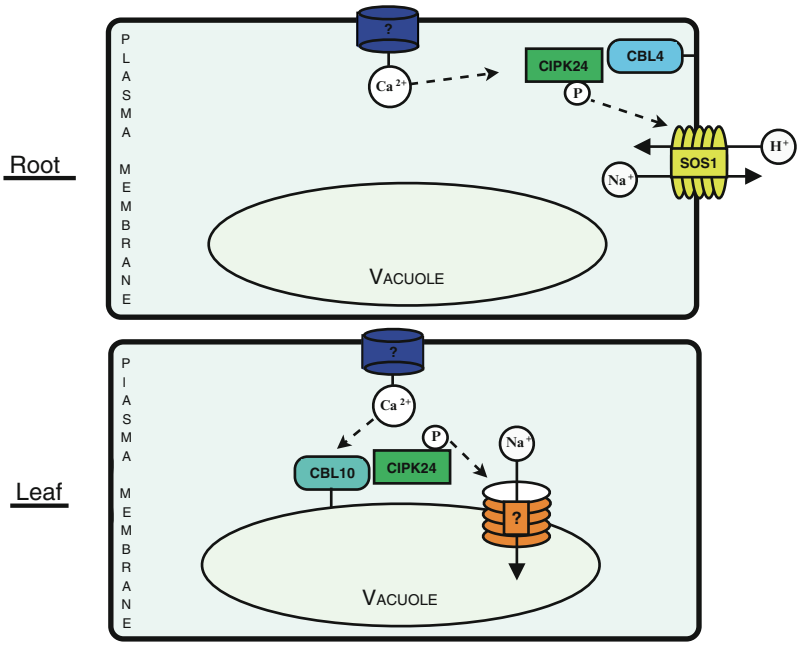

Fig. 4 A model of CBL–CIPK function in salt stress response. In root cells, CIPK24 activates the Na^+/H^+ antiporter SOS1 at the plasma membrane by forming a complex with CBL4. In leaf cells, CIPK24 regulates Na^+ sequestration into the vacuole by interacting with the tonoplast-bound CBL10

5 Conclusions

Research during the last few decades has revealed that plant signal transduction pathways are complex and highly intertwined, which perhaps reflects the challenges of responding to the dynamic conditions of most terrestrial environments. In order to flourish in the variable environments in which they reside, plants must decide on appropriate courses of action when faced with environmental perturbations and stresses, which often occur as combinations of multiple forms of stress. Calcium signaling has emerged as an integral process that enables plants to process numerous signals of their environment and coordinate vital responses. The CBL–CIPK network is an illustrative example of our understanding of the workings of plant calcium signaling pathways. CBL–CIPK network members have been shown to act as convergence points for distinct signals, as is the case for Arabidopsis CIPK23, which participates in both potassium and nitrate responses. CIPK23 itself is regulated by CBL1 and CBL9, which serve to integrate ABA-independent and ABA-dependent signals. Other network members act as master switches that coordinate multiple downstream processes to achieve effective environmental responses, as is the case for Arabidopsis CIPK24, which through alternative

interactions with CBL4 or CBL10 activates transporters that extrude toxic sodium ions from the cytoplasm to the apoplast or the vacuole, respectively, and appears to regulate the activity of multiple transporters involved in high-salt responses.

Despite the many examples of CBL and CIPK functions presented here, the functions of most CBLs and CIPKs from Arabidopsis remain uncharacterized to date. Moreover, our understanding of the CBL–CIPK network in other higher plants remains fragmentary at best, and the functions of computationally identified CBLs and CIPKs from early diverging land plants and green algae are completely unknown. It is intriguing that the CBL–CIPK networks of higher plants contain many more members than the networks of basal plants and green algae; however, the significance of this observation is unclear. In addition to the acquisition of novel functions, it is probable and in some cases demonstrated that CBLs and CIPKs from higher plants such as Arabidopsis have partially overlapping functions, and this has likely been an impediment to the discovery of new functions of CBLs and CIPKs. For this reason, it is hopeful that studies of multiple knock-out mutants and RNAi lines with reduced expression of multiple CBLs and CIPKs will lead to further discoveries. Furthermore, investigation of the functionality of the CBL–CIPK network in other model organisms will complement insights gained from experiments using Arabidopsis. By developing a deeper understanding of the functions and mechanisms of the CBL–CIPK network, researchers are beginning to elucidate how plants have co-opted calcium signals to serve as the keystone messengers of their environment.

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