

Integrated Calcium Signaling in Plants

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Abstract Calcium ion (Ca^{2+}) is the most important universal signal carrier used by living organisms, including plants, to convey information to many different cellular processes. The cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) has been found to increase in response to many physiological stimuli, including stress. The Ca^{2+} spikes normally result from two opposing reactions, Ca^{2+} influx through channels or Ca^{2+} efflux through pumps. The removal of increased Ca^{2+} from the cytosol to either the apoplast or intracellular organelles requires energized “active” transport. Ca^{2+} -ATPases and $\text{Ca}^{2+}/\text{H}^{+}$ antiporters are the key proteins catalyzing this movement. The increased level of Ca^{2+} is recognized by some Ca^{2+} sensors or calcium-binding proteins, which can activate many calcium-dependent protein kinases. The regulation of gene expression by cellular Ca^{2+} is also crucial for plant defense against various stresses. In this chapter several aspects of calcium signaling, such as Ca^{2+} requirement, Ca^{2+} transporters/pumps (Ca^{2+} -ATPases, $\text{Ca}^{2+}/\text{H}^{+}$ antiporter), Ca^{2+} signature, Ca^{2+} memory, and various Ca^{2+} -binding proteins, are presented.

1 Introduction

The maintenance of intracellular ionic homeostasis is fundamental to the physiological function of a living cell. The Ca^{2+} is a versatile signal ion regulating many physiological processes, such as ionic balance, gene expression, metabolism of lipids, proteins, and carbohydrates, cell growth, cell division, cell development, and secretion (Poovaiah and Reddy 1993; Pardo et al. 1998; Mahajan et al. 2006a; Tuteja and Mahajan 2007; Tuteja 2007). Many external stimuli, including light and various stress factors, can bring about changes in cellular Ca^{2+} level, which can affect plant growth and development (Sanders et al. 2002; Rudd and Franklin-Tong 2001). Ca^{2+} serves as a second messenger and its concentration is delicately balanced by the presence of “ Ca^{2+} stores” such as vacuoles, endoplasmic reticulum (ER), mitochondria,

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and cell wall. Recently, Xiong et al. (2006) demonstrated that the organelles surrounded by a double membrane (e.g., mitochondria, chloroplasts, and nuclei) are also equipped to generate calcium signal on their own, which is delimited by a double membrane. Ca^{2+} ion represents an important signaling species and a convergence point of many disparate signaling pathways. A generic signal transduction pathway has the following steps: (1) perception of the signal by the membrane receptors, (2) generation of second messengers, (3) a cascade of protein phosphorylation/dephosphorylation events that may target transcription factors controlling a specific set of stress-regulated genes, and (4) stress tolerance, plant adaptation, and other phenotypic responses (Tuteja and Mahajan 2007).

In plants, Ca^{2+} plays many essential roles. For example, it is an essential plant nutrient required for growth and development, especially of the root and the shoot tip, which are meristematic, where cell division occurs by mitosis. Ca^{2+} helps in the formation of microtubules and microtubules in turn are essential for the anaphasic movement of chromosomes. Ca^{2+} is an important divalent cation and is required for structural roles in the cell wall and membranes, where it exists as calcium pectate. Ca^{2+} accumulates as calcium pectate in the cell wall and binds the cells together. It is also required as a counteranion for inorganic and organic anions in the vacuole and as an intracellular messenger in the cytosol (Mahajan and Tuteja 2005). Ca^{2+} is required for pollen tube growth and elongation (Sanders et al. 2002). Several genes are reported to be upregulated in response to calcium in plants. An analysis of transcriptome changes revealed 230 calcium-responsive genes, of which 162 were upregulated and 68 were downregulated (Kaplan et al. 2006). These include known early stress-responsive genes as well as genes of unknown function. Recently, a blue light receptor phototropin, which regulates growth and development of plants, has also been shown to be involved in calcium signaling in higher plants (Harada and Shimazaki 2007). The Ca^{2+} signaling pathway can also regulate a K^+ channel for low- K response in *Arabidopsis*. Calcium is also reported to be an essential component of the sucrose-signaling pathway that leads to the induction of fructan synthesis (Martinez-Noel et al. 2006). Calcium signaling is also involved in the regulation of cell cycle progression in response to abiotic stress. In appreciation of the immense significance of Ca^{2+} ions, this chapter is solely dedicated to the salient features associated with integrated calcium signaling. Various aspects regarding the Ca^{2+} requirements of plant and signal-induced changes, Ca^{2+} transporters/efflux pumps, $\text{Ca}^{2+}/\text{H}^+$ antiporters, Ca^{2+} signatures, Ca^{2+} memory, and Ca^{2+} sensor and transducer proteins are briefly covered.

2 Ca^{2+} Concentrations in Plant Cells and Signal-Induced Changes

Ca^{2+} is also an essential plant macronutrient element which is taken up by roots from the soil and is delivered to the shoot via xylem. In general, the cytosolic Ca^{2+} , typically kept at submicromolar levels in plant cells, is a well-established link for various signal transduction networks (Poovaiah and Reddy 1993; Bush 1995). The cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) concentration in plant cells is usually

maintained at 200 nM (Bush 1995) or in the range of 0.1–1 μ M (Poovaiah and Reddy 1993). However, the $[Ca^{2+}]_{cyt}$ is far higher than this because of the high affinity for Ca^{2+} of a range of Ca^{2+} -binding proteins. With use of various techniques it has been shown that Ca^{2+} concentrations in apoplasts range between 0.1–1.0 and 1–3 mM in ER lumen and these levels can go up to 50 mM (Sarwat and Tuteja 2007). Plastids and mitochondria contain millimolar levels of Ca^{2+} but much less than ER. Ca^{2+} concentrations within the vacuole range from 0.1 to 10 mM. In sieve tubes, the Ca^{2+} level is reported to be 20–100-fold higher than it is in typical plant cells (Knoblauch et al. 2001). Plants vary in their Ca^{2+} requirements and the ability to extract Ca^{2+} from complex soil environments. In particular, monocots require less Ca^{2+} for optimal growth than do dicots (see Tuteja and Mahajan 2007).

The environmental and hormonal signals are known to induce changes in cytosolic Ca^{2+} (Poovaiah and Reddy 1993). Regulation of Ca^{2+} level is important for the survival of the cell. The plant cell contains a number of vesicular compartments, which store Ca^{2+} that can be released into the cytoplasm when required. Specific channels/pumps regulate the movement of Ca^{2+} in and out of cells and organelles (Mahajan et al. 2006a; Tuteja 2007). While the source of Ca^{2+} resulting in its increase in the cytosol after a signal has not been extensively studied, in general we know that Ca^{2+} release can be primarily from an extracellular source (apoplastic space) as addition of ethylene glycol bis(2-aminoethyl ether) *N,N,N',N'*-tetraacetic acid or 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid was shown in many cases to block calcium effects (Fig. 1). Ca^{2+} release may also result from activation of PLC, leading to hydrolysis of phosphatidylinositol bisphosphate to inositol triphosphate (IP_3) and subsequent release of Ca^{2+} from intracellular Ca^{2+} stores (Fig. 1). In plants, resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillates diurnally. Tang et al. (2007) reported that in *Arabidopsis thaliana*, $[Ca^{2+}]_i$ oscillations are synchronized to extracellular Ca^{2+} concentration oscillations largely through the Ca^{2+} -sensing receptor CAS. CAS regulates concentrations of IP_3 , which in turn directs release of Ca^{2+} from internal stores. Furthermore, calcium-binding proteins (calcium sensors) can provide an additional level of regulation in the calcium signaling. These sensor proteins recognize and decode the information provided in the calcium signatures and relay the information downstream to initiate a phosphorylation cascade, leading to the activation of the signal-induced genes, products of which can directly or indirectly provide the signal response (e.g., stress tolerance). The signal response could also be growth inhibition or cell death, which will depend upon what kinds of genes are upregulated or downregulated (Mahajan et al. 2006a; Tuteja 2007) (Fig. 1). Overall, the calcium-induced response could be a coordinated action of many genes.

Mechanical stimuli (wind and touch) and fungal elicitors have also been reported to induce rapid and transient increases in cytosolic Ca^{2+} (Poovaiah and Reddy 1993). The source for the increase in cytosolic Ca^{2+} by different signals may be different, for example, the source for cold signal is extracellular and that for wind signal is intracellular. In response to abscisic acid (ABA) an increase (37–80%) in cytosolic Ca^{2+} in the opened stomatal guard cells has been observed. Light (phototropism) and gravity (geotropism) in maize coleoptiles have been shown to be responsible for increasing the cytosolic Ca^{2+} . Gibberellic acid increases the cytosolic Ca^{2+} of

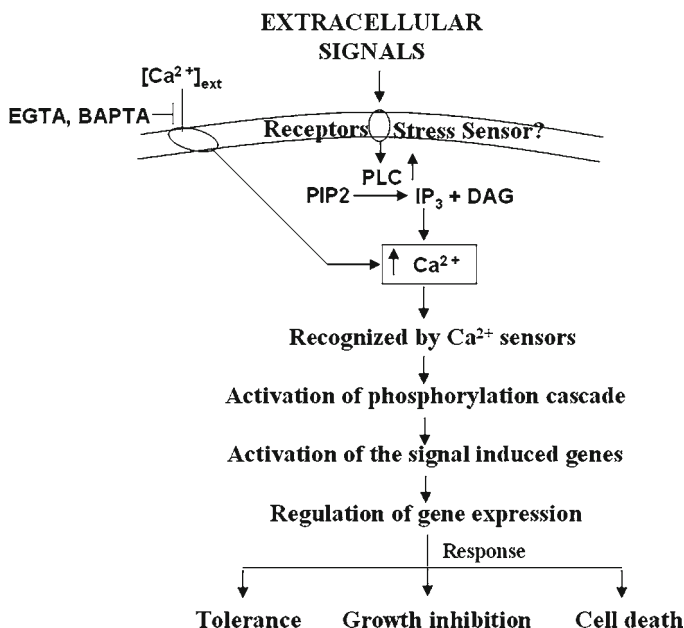


Fig. 1 Generic pathway for calcium signaling. The extracellular stress signal is first perceived by the membrane receptors and then activates a large and complex signaling cascade intracellularly including the generation of secondary signal molecules. High-salinity (Na^+) stress initiates the calcium signaling network. The signal first activates phospholipase C, which hydrolyzes phosphatidylinositol biphosphate to generate inositol triphosphate and diacylglycerol, resulting in an increase in the level of Ca^{2+} ions. This increase in Ca^{2+} ion concentration is sensed by calcium sensors, which interact with and activate some protein kinases. The signal cascade results in the expression of multiple stress-responsive genes, the products of which can provide the phenotypic response of stress tolerance directly or indirectly. The stress response could also be a growth inhibition or cell death, which will depend upon how many and what kinds of genes are upregulated or downregulated. Overall, the stress response could be the coordinated action of many genes which may talk with each other

barley aleurone protoplast threefold (50–150 mM), while ABA decreases the cytosolic Ca^{2+} of barley aleurone protoplast (for all the above, see Poovaiah and Reddy 1993). Blue light is also known to increase cytosolic Ca^{2+} . Recently, Chena et al. (2008) suggested that inositol polyphosphate 5-phosphatase-13, a key enzyme in the phosphatidylinositol metabolic pathway, antagonizes PHOT1-mediated effects on calcium signaling under blue light.

3 Calcium Transporters/Pumps

Active transport of Ca^{2+} from the cytosol is a prerequisite for the restoration of low levels cytosolic calcium after the signaling event. Ca^{2+} -ATPases and $\text{Ca}^{2+}/\text{H}^+$ antiporters are the key proteins catalyzing this movement. By the removal of Ca^{2+} from the cytosol several important functions are performed by these proteins (Hirschi 2001). Some of the functions are as follows:

1. These proteins maintain a low cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in the resting or the unstimulated cell which is appropriate for the cytoplasmic metabolism.
2. These proteins restore $[\text{Ca}^{2+}]_{\text{cyt}}$ levels to the resting normal levels following a $[\text{Ca}^{2+}]_{\text{cyt}}$ perturbation, thereby influencing the kinetics of the magnitude and subcellular location of $[\text{Ca}^{2+}]_{\text{cyt}}$ signals.
3. These proteins replenish intracellular and extracellular Ca^{2+} stores for subsequent $[\text{Ca}^{2+}]_{\text{cyt}}$ signals and permit the generation of localized $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations through their interplay with Ca^{2+} channels (Harper 2001).
4. These proteins provide Ca^{2+} in the ER for the secretory system to function.
5. These proteins remove some divalent cations, such as Ni^{2+} , Zn^{2+} , Mg^{2+} , and Mn^{2+} , from the cytosol to prevent mineral toxicity (Hirschi 2001).

Hirschi (2001) suggested that the Ca^{2+} -ATPases, which have high affinity ($K_m = 1\text{--}10\ \mu\text{M}$) but low capacity for Ca^{2+} transport, are responsible for maintaining $[\text{Ca}^{2+}]_{\text{cyt}}$ homeostasis in the resting cells, whereas the $\text{Ca}^{2+}/\text{H}^+$ antiporters, which have lower affinities ($K_m = 10\text{--}15\ \mu\text{M}$) but high capacities for Ca^{2+} transport, are likely to remove Ca^{2+} from the cytosol during $[\text{Ca}^{2+}]_{\text{cyt}}$ signals and thereby modulate $[\text{Ca}^{2+}]_{\text{cyt}}$ perturbations. This hypothesis is supported by the fact that the $\text{Ca}^{2+}/\text{H}^+$ antiporter, but not the vacuolar Ca^{2+} -ATPase, resets $[\text{Ca}^{2+}]_{\text{cyt}}$ in yeast following hypertonic shock (Denis and Cyert 2002).

3.1 Ca^{2+} -ATPases

Ca^{2+} -ATPases are estimated to represent less than 0.1% of the membrane protein and are thus 30–100-fold less abundant than H^+ -ATPases in the plasma membrane (3%) and the endomembranes (5–10%). ATPase pumps are condemned to push Ca^{2+} uphill for eternity into the ER or out of the cell. Plant Ca^{2+} -ATPases belong to two major families: (1) the P-type ATPase IIA family and (2) the P-type ATPase IIB family (Axelsen and Palmgren 2001).

3.1.1 P-Type ATPase IIA Family

The nucleotide specificity of these pumps is broad (30–60% activity achieved with GTP and ITP). The pumps are inhibited by erythrosine B ($\text{IC}_{50} \leq 1\ \mu\text{M}$) and Ca^{2+} affinity is estimated to be in the range of 0.4–12 μM . The first family. The P-type ATPase II A family lacks an N-terminal autoregulatory domain. Four members of this family have been identified in the *Arabidopsis* genome (termed “AtECAs 1–4” by Axelsen and Palmgren (2001). They are likely to be present in the plasma membrane, tonoplast ER, and the Golgi apparatus.

3.1.2 P-Type ATPase IIB Family

This second family is characterized by an autoinhibitory N-terminal domain that contains a binding site for Ca-calmodulin (CaM) and in addition a serine-residue

phosphorylation site. Their catalytic activity can be modulated by $[Ca^{2+}]_{cyt}$ either through activation upon binding CaM or by inhibition following phosphorylation by Ca^{2+} -dependent protein kinases (CDPKs) (Hwang et al. 2000). Since CaM binding sites are generally quite diverse, each type IIB Ca^{2+} -ATPase may have different affinity for CaM or may bind a different CaM isoform. Ten members of the type IIB Ca^{2+} -ATPase family have been identified in the *Arabidopsis* genome and are termed “ACASs 1, 2, and 4” and “ALACAs 7–13” (Axelsen and Palmgren (2001). These Ca^{2+} -ATPases reside on various cellular membranes, including the plasma membrane (AtACA8), the tonoplast (AtACA4), and the plastid inner membrane (AtACA1). The relative molecular mass of type IIB Ca^{2+} -ATPase pumps has been estimated to be between 115,000 and 135,000Da (Rasi-Caldogno et al. 1995).

The abundance of Ca^{2+} -ATPase isoforms suggests that individual isoforms are functionally distinct and may respond differentially to distinct cellular processes involving specific Ca^{2+} signals. It also implies a requirement for CaM-independent and CaM-dependent regulation of Ca^{2+} -ATPase activities in the modulation of $[Ca^{2+}]_{cyt}$ perturbations during cell signaling. The expression of many Ca^{2+} -ATPases is increased upon exposure to high salinity or high $[Ca^{2+}]_{cyt}$ and some Ca^{2+} -ATPase genes are expressed only under stress conditions (Garcia-deblas et al. 2001). This may reflect a role in maintaining $[Ca^{2+}]_{cyt}$ homeostasis or in reducing Na^+ influx to the cytosol in saline environments.

3.2 Ca^{2+}/H^+ Antiporter

Calcium regulation of ion homeostasis by various ion pumps is depicted in Fig. 2. Ca^{2+}/H^+ antiporters are efflux transporter and are different from Ca^{2+} -ATPases in that they do not require ATP and they are not sensitive to vanadate. *CAX1* (calcium exchanger 1) was the first plant Ca^{2+}/H^+ antiporter which was cloned and functionally expressed (Tuteja and Mahajan 2007). The gene was identified by its ability to restore growth on a high Ca^{2+} medium to a yeast mutant defective in vacuolar Ca^{2+} transport. The Ca^{2+}/H^+ antiporters present in the plasma membrane and tonoplast have been characterized biochemically (Sanders et al. 2002). These have a lower affinity for Ca^{2+} than Ca^{2+} -ATPases and may also transport Mg^{2+} . Eleven genes encoding putative Ca^{2+}/H^+ antiporters (*AtCAX*) have been identified in the genome of *Arabidopsis thaliana* (Hirschi 2001). The transporters *AtCAX1*, *AtCAX2*, and *AtCAX4* are located at the tonoplast. The *AtCAX1* antiporter exhibits both high affinity and high specificity for Ca^{2+} , whereas the *AtCAX2* transporter is a high-affinity, high-capacity H^+ /heavy metal cation antiporter. The *AtCAX* genes have homologues in other plant species and their physiological roles have been investigated using transgenic plants (Hirschi 2001). Transgenic tobacco overexpressing *AtCAX1* exhibits Ca^{2+} -deficiency disorders, which include tip burn, metal hypersensitivity, and susceptibility to chilling, that can be reversed by increasing Ca^{2+} supply. Increase of the Ca^{2+} supply resulted in the expression of *AtCAX1* and *AtCAX3* (but not *AtCAX2* or *AtCAX4*) genes being increased (Hirschi 2001). Ca^{2+}/H^+ antiporters utilize the H^+ gradient generated by the tonoplast V-type H^+ pump and by a

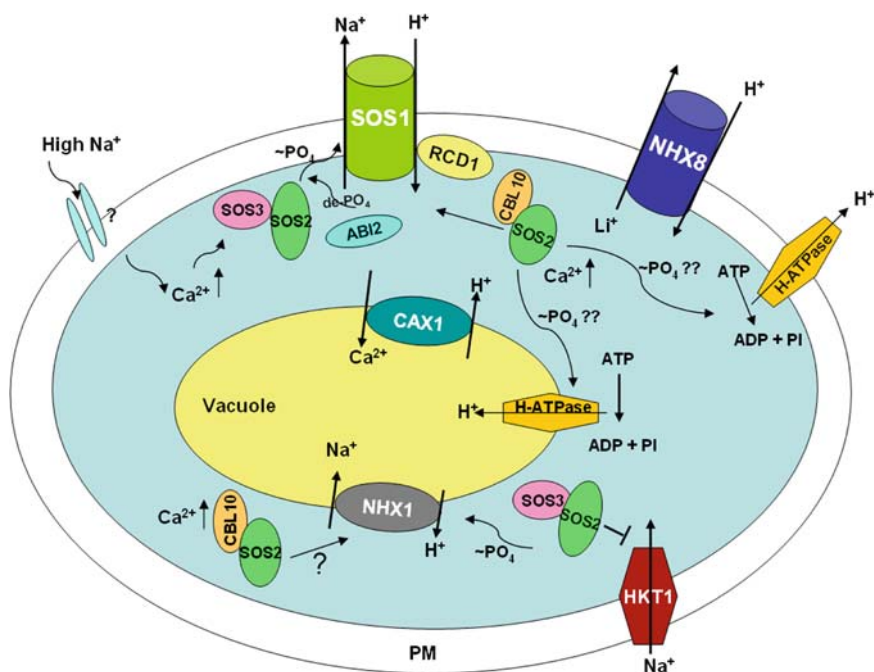


Fig. 2 Calcium and SOS pathway in regulation of ion homeostasis by various ion pumps. Under saline condition, the increase in the cytosolic Na^+ level causes damage to several cellular processes. One of the major salt detoxifying mechanisms in the cell is the calcium-activated SOS3-SOS2 protein complex, which activates SOS1, a Na^+/H^+ antiporter on the plasma membrane responsible for extrusion of Na^+ from the cell. At the same time the SOS3-SOS2 complex is also involved in inhibiting HKT1, low-affinity potassium transporter, which transports Na^+ ion under high salt condition. Recently, another member of the SOS3 family, CBL10, has also been found to form a complex with SOS2. This complex is speculated to regulate both extrusion of Na^+ ion (by regulating SOS1) and sequestration/compartimentalization of Na^+ ion into the vacuole (activating the NHX-type transporter which pumps Na^+ ion into the vacuole). SOS1 also interacts with RCD1 and imparts protection against reactive oxygen species and oxidative stress. SOS2 also works to activate H^+ -ATPases under salt stress and helps in reinstating ionic homeostasis. For details, refer to the text and Mahajan et al. (2008)

proton-pumping pyrophosphatase to sequester Ca^{2+} in the vacuole (Tuteja and Mahajan 2007). In many plant cells the vacuole occupies more than 50% of the cell volume, and it is evident that *trans* tonoplast Ca^{2+} transport makes a very significant contribution to the regulation of cytosolic Ca^{2+} concentrations.

4 Ca^{2+} Signature

It is now clear that transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ is a universal response to stress and also critical for the production of a physiological response. Perturbation in cytosolic calcium levels (termed “ $[\text{Ca}^{2+}]_{\text{cyt}}$ signature”) is unique and results in an appropriate

physiological response to a particular stimulus. The uniqueness is manifested in the subcellular location and/or the kinetics of the magnitude of the $[Ca^{2+}]_{cyt}$ perturbation (Rudd and Frankin-Tong 2001). The transient increase in $[Ca^{2+}]_{cyt}$ can be single (spike), double (biphasic), or multiple (oscillations). The perturbations generated may differ in their cellular location, role, and extent of propagation and in their amplitude during propagation. Several abiotic challenges result in an immediate, transient increase in $[Ca^{2+}]_{cyt}$ that is restored to basal levels within minutes. Such changes include mechanical perturbations and rapid cooling for brief periods, termed “cold shock.” The duration, periodicity, and amplitude of oscillations vary considerably, and their form is often dependent on the strength and combination of specific stimuli (Allen et al. 1999). Calcium signatures may also be tissue-specific. For example, within the root, the $[Ca^{2+}]_{cyt}$ perturbations induced by mechanical perturbation, salinity, osmotic stress, cold shock, or slow cooling differ markedly between cell types (Moore et al. 2002).

An increase in $[Ca^{2+}]_{cyt}$ is effected by Ca^{2+} influx to the cytosol either from the apoplast, across the plasma membrane, or from the intracellular organelles. The Ca^{2+} influx is mediated by Ca^{2+} permeable ion channels, and their type, cellular localization, and abundance influences the spatial characteristics of $[Ca^{2+}]_{cyt}$ perturbations. Since the diffusion of Ca^{2+} within the cytoplasm is low, and the buffering of Ca^{2+} in the cytoplasm is high (0.1–1 mM) (Trewavas 1999), the opening of the Ca^{2+} channel produces a local increase in $[Ca^{2+}]_{cyt}$ that dissipates rapidly after the channel has been closed. The subcellular localization of the Ca^{2+} channel is therefore critical for the targeting of different cellular processes. Cytosolic calcium “waves” are produced within the cytoplasm by the successive recruitment of particular Ca^{2+} channels to coordinate cellular responses. It was suggested that a local elevation of $[Ca^{2+}]_{cyt}$ might generate soluble second messengers, such as IP_3 or cADPR (cyclic ADP Ribose), that diffuse through the cytoplasm to activate a relay of spatially separated Ca^{2+} channels (Trewavas 1999). This theory was supported by plant cells responding to salt stress (Tuteja and Mahajan 2007). In addition to these subcellular waves, “waves” of cells with high $[Ca^{2+}]_{cyt}$ may also propagate through the plant tissue. This can be induced in root tissues by mechanical stimulation or saline shock, in cotyledons by cold shock, and in leaves by chilling plant roots briefly. Electrical action potentials, osmotic perturbations, or chemical signals may trigger these waves. Although elevated $[Ca^{2+}]_{cyt}$ is necessary for signal transduction, a prolonged increase in $[Ca^{2+}]_{cyt}$ is lethal. Sustained high $[Ca^{2+}]_{cyt}$ is implicated in apoptosis, both during the normal development and in hypersensitive responses to pathogens (Tuteja and Mahajan 2007).

5 Ca^{2+} Memory

There is considerable evidence that $[Ca^{2+}]_{cyt}$ signatures are modified by previous experience. A diminished $[Ca^{2+}]_{cyt}$ elevation upon repetitive stimulation by the same environmental challenge or a developmental cue is a common observation. All these suggested the existence of Ca^{2+} memory in plant cells. The term

“memory” was put forward first by Knight et al. (1996). The attenuated response of $[Ca^{2+}]_{cyt}$ after repeated stimulation by various elicitors forms a part of cellular memory and the cells are able to retain the previous information. This memory is significant and helps the cells respond better to a particular stress without disturbing the delicate balance of Ca^{2+} levels. Some examples to support this are as follows:

1. The magnitude of the $[Ca^{2+}]_{cyt}$ perturbation elicited by the wind-induced motion becomes progressively smaller upon repeated stimulation and a refractory period of several minutes is required before a full response is observed again. A second exposure to an elicitor does not influence $[Ca^{2+}]_{cyt}$ for several hours after its initial application (Blume et al. 2000).
2. Plant cells challenged with H_2O_2 fail to respond to H_2O_2 for several hours (Price et al. 1994).
3. There is also evidence that the $[Ca^{2+}]_{cyt}$ signatures elicited by one environmental challenge can be modified by prior exposure to a contrasting one. For example, the magnitude of the $[Ca^{2+}]_{cyt}$ perturbations in response to oxidative stress was reduced by prior exposure to hyperosmotic stress and the opposite was also found to be true. These observations also imply cross talk between the signaling cascades.

6 Calcium-Binding Proteins

In general, Ca^{2+} binding triggers changes in protein shape and charge. Several reports indicate that Ca^{2+} regulates the transcription of target genes by affecting changes in the phosphorylation status of specific transcription factors. ABA-induced expression of two genes in aurea mutants of tomato, *rd-29A*, a desiccation-induced gene, and *kin2*, a cold-responsive gene, is also reported to be Ca^{2+} -regulated. Analysis of transcriptome changes in response to increase in cytosolic Ca^{2+} revealed that ABA response element related sequences also act as calcium-responsive *cis* elements (Kaplan et al. 2006). It seems therefore that Ca^{2+} can control many cellular processes in plant cells by interacting with various proteins and kinases, which results in the regulation of expression of various genes. Recently a novel family of transcription factors that bind CaM, named “CaM-binding transcription activators,” have been identified and indicated to be involved in stress-induced calcium-mediated gene expression in *Arabidopsis* (Bouché et al. 2002; Yang and Poovaiah 2002).

An increase level of the cytoplasmic Ca^{2+} in response to signals is sensed by an array of Ca^{2+} sensors, which are small Ca^{2+} -binding proteins that change their conformation in a Ca^{2+} -dependent manner. Specificity in the signaling pathway is provided by the uniqueness in calcium signatures and also by plethora of Ca^{2+} sensors, which can decode the Ca^{2+} perturbations quite precisely. Once Ca^{2+} sensors have decoded the elevated $[Ca^{2+}]_{cyt}$, Ca^{2+} efflux into the cell exterior and/or the sequestration into cellular organelles such as vacuoles, ER, and

mitochondria to restore its level to that of the resting state. The EF-hand is the most frequent motif found in Ca^{2+} -binding proteins. There are at least two types of Ca^{2+} -binding proteins, one which contains EF-hand and other which does not contain EF-hand.

6.1 *EF-Hand Containing Ca^{2+} -Binding Proteins (Ca^{2+} Sensors)*

Most of the Ca^{2+} sensors bind Ca^{2+} using a helix-loop-helix motif termed the “EF hand” (named after the E and F regions of parvalbumin), which binds a single Ca^{2+} ion with high affinity (Tuteja and Mahajan 2007). The Ca^{2+} sensors utilize the side-chain oxygen atoms of the EF-hand motif for Ca^{2+} coordination. In 1973 Kretsinger and Nockolds (1973) first discovered the EF-hand structural motif in the crystal structure of parvalbumin. The properties of EF-hands are described below:

1. The EF-hand motifs are mostly found back-to-back in antiparallel pairs with β -sheet-like hydrogen-bonding occurring between the loops of the coupled sites. These motifs help in the stabilization of the protein structure.
2. The EF-hand is a highly conserved 29 amino acid motif consisting of an α helix E (residues 1–10), a loop (residue 10–21), which binds the Ca^{2+} ion, and a second α -helix F (residues 19–29).
3. The Ca^{2+} ion is coordinated by an oxygen atom or by a bridging water molecule of the side chains (Tuteja and Mahajan 2007).
4. In most of the functional EF-hand motifs, the first amino acid is aspartate and the 12th is glutamate. Glutamate contributes both its side-chain oxygen atoms to the metal ion coordination.
5. Most of the EF-hand proteins are characterized by the relatively high percentage of acidic residues.
6. Several isoforms of an EF-hand protein may exist in a single organism.

The Ca^{2+} binding affinities of the EF-hand protein vary substantially ($K_d=10^{-4}$ – 10^{-9}M) and depend on the amino acid sequence of the protein, especially with regard to the 12-residue consensus loop that provides all the acids that directly ligate to Ca^{2+} ions. There are many EF-hand-containing calcium sensors in plants. The major families of Ca^{2+} sensors include CaM, CaM-like proteins, CDPKs, and calcineurin B-like proteins (CBL). These are briefly described next.

6.1.1 **Calmodulin**

CaM (17 kDa) is a prototypical Ca^{2+} -sensor protein that can control many important biological functions by binding to hundreds of target proteins. It is a highly conserved acidic protein with two globular domains, each containing two EF-hands, connected by a flexible α -helical linker (Luan et al. 2002). CaM is found in the apoplast, the cytosol, the ER and the nucleus of plant cells. The specific biological functions of plant CaM are not well known. Yoo et al. (2005)

isolated a complementary DNA (cDNA) encoding a CaM-binding transcription factor, MYB2, that regulates the expression of salt- and dehydration-responsive genes in *Arabidopsis*. CaM works in a calcium-dependent or calcium-independent manner by binding to and regulating the activity of target proteins called “CaM-binding proteins.” Protein phosphatase PP7 is the first protein serine/threonine phosphatase to be found to interact with CaM in plants. PP7 is reported to be involved in thermotolerance in *Arabidopsis* (Liu et al. 2007). Katou et al. (2007) have shown that a CaM-binding mitogen-activated protein kinase phosphatase is induced by wounding and regulates the activities of stress-related mitogen-activated protein kinases in rice. Within the cytosol, the estimated CaM concentration is 5–40 μM (Zielinski 1998; Rudd and Frankin-Tong 2001). The role of CaM has been implicated in many physiological processes, such as those affected by light, gravity, mechanical stress, phytohormones, pathogens, osmotic stress, heat shock, and chilling (Zielinski 1998; Rudd and Frankin-Tong 2001). The structure of CaM revealed that all four EF-hands are saturated by Ca^{2+} ions. CaM appears to be regulatory protein and induces large changes in interhelical angles as Ca^{2+} is bound. The affinity of CaM for Ca^{2+} is influenced by the presence of particular target proteins (Zielinski 1998). CaM can also regulate gene expression by binding to specific transcription factors (Bouché et al. 2002).

6.1.2 CaM-Like Proteins

Plants also possess CaM-like proteins, which differ from CaM in containing more than 148 amino acid residues and have between one and six EF-hand motifs. They possess limited homology to CaM (75% identity) with canonical CaM isoforms (Luan et al. 2002). In *Arabidopsis*, they include CaBP-22, TCH2, TCH3, AtCP1, NADPH oxidases, and Ca^{2+} binding protein phosphatases such as ABI 1 and ABI 2. These proteins have been implicated in cellular responses to diverse environmental, developmental, and pathological challenges.

6.1.3 Ca^{2+} -Dependent Protein Kinases

Five different types of Ca^{2+} regulated protein kinases have been reported in plants. These include (1) CDPKs independent of CaM, (2) CDPK-related protein kinases (CRKs), (3) CaM-dependent protein kinases (CaMKs), (4) Ca^{2+} /CaM-dependent protein kinases (CCaMK), and (5) SOS3/CBL interacting protein kinases (SIPKs/CIPKs).

1. CDPKs independent of CaM. There are at least 34 genes encoding CDPKs in the *Arabidopsis* genome (Cheng et al. 2002) and similar numbers in other plant species. These CDPKs are ubiquitous in plants and generally have four EF-hands at their C-terminus that bind Ca^{2+} and activate the serine/threonine

kinase activity of the enzyme. These kinases require micromolar concentrations of Ca^{2+} for their activity and have no requirement for CaM or lipids. They have a unique structure as the N-terminal protein kinase domain is fused with the C-terminal autoregulatory domain and a CaM-like domain, which has a Ca^{2+} -binding EF-hand or helix-loop-helix motif. The autoinhibitory domain of CDPKs is a 30 amino acid sequence, which acts as a pseudo-substrate (Harper et al. 1994). The N-terminal domain of CDPKs is variable and provides specificity to different CDPK isoforms. These enzymes show several-fold stimulation with Ca^{2+} and show autophosphorylation. The binding of Ca^{2+} to some of CDPKs is modulated by lipids or phosphorylation (Cheng et al. 2002). Ca^{2+} binding to CDPK effects conformation of the kinase and relieves the inhibition caused by the autoinhibitory region. CDPKs are implicated in pollen development, control of cell cycle, phytohormone signaling, light-regulated gene expression, gravitropism, thigmotropism, cold acclimation, salinity tolerance, drought tolerance, and responses to pathogens (Xiong et al. 2002).

2. *CRKs*. CRKs are similar to CDPKs except that the CaM-like region is poorly conserved with degenerate or truncated EF-hands that may not be able to bind Ca^{2+} . There are at least seven CRKs in the *Arabidopsis* genome, and orthologues of these are present in many plant species. However, the regulation and function of these kinases are not known (Harmon et al. 2001).
3. *CaMKs*. Several CaMKs have been cloned from *Arabidopsis* and other plants. Kinase activity of CaMKs is stimulated by CaM-dependent autophosphorylation and their catalytic activity is also modulated by CaM. They are highly expressed in rapidly growing cells and tissues of the root and flower (Zhang and Lu 2003). Recently, Liu et al. (2008) showed that CaM-binding protein kinase 3 is part of heat shock signal transduction in *Arabidopsis thaliana*.
4. *CCaMKs*. These are a group of Ca^{2+} -dependent kinases which in addition to Ca^{2+} also require CaM for their activity. Thus, CaM besides acting directly could also exert its effect by binding to protein kinases and modulating their activity. A CCaMK was characterized from lily and other plant species (Lu et al. 1996). Sequence analysis revealed the presence of an N-terminal catalytic domain, a centrally located CaM-binding domain, and a C-terminal visinin-like domain containing only three EF-hands. Biochemical studies of CCaMK established that Ca^{2+} and CaM stimulate CCaMK activity. In the absence of CaM, Ca^{2+} promotes autophosphorylation of CCaMK. The phosphorylated form of CCaMK possesses more kinase activity than the nonphosphorylated form. Recently, Jeong et al. (2007) reported a novel *Arabidopsis* CCaMK (AtCK) which is presumably involved in CaM-mediated signaling.
5. *SIPKs/CIPKs*. CBL were found to interact specifically with a class of serine/threonine protein kinases known as CIPKs (Mahajan and Tuteja 2005; Mahajan et al. 2006a, 2008). Recently, a novel CIPK from pea was reported and was found to interact and phosphorylate the pea CBL (Mahajan et al. 2006b). Genetic analysis confirmed that SOS1-SOS3 function in a common pathway of salt tolerance. This pathway also emphasizes the significance of Ca^{2+} signal in reinstating cellular ion homeostasis. The SOS pathway is depicted in Fig. 2.

The increased level of cytosolic Ca^{2+} caused by salt stress is sensed by a calcium sensor such as SOS3, which interacts with SOS2 protein kinase. This SOS3-SOS2 protein kinase complex phosphorylates SOS1, a plasma membrane Na^+/H^+ antiporter, resulting in efflux of excess Na^+ ions (Mahajan et al. 2008). This complex interacts with and influences other salt-mediated pathways, resulting in ionic homeostasis. This complex inhibits HKT1 activity (a low-affinity Na^+ transporter), thus restricting Na^+ entry into the cytosol. SOS2 also interacts and activates NHX (vacuolar Na^+/H^+ exchanger), resulting in sequestration of excess Na^+ ions, further contributing to Na^+ ion homeostasis. In a cell, phosphorylation events are often coupled with dephosphorylation events for the maintenance of homeostasis. SOS2 also interacts with ABI2. It is possible that ABI2 may dephosphorylate the proteins that are phosphorylated by SOS2 in order to restore homeostasis after a stress condition. CAX1 ($\text{Ca}^{2+}/\text{H}^+$ antiporter) has been identified as an additional target of SOS2 activity reinstating cytosolic Ca^{2+} homeostasis under salt stress (Fig. 2). Research has led to further elucidation of this classic SOS pathway. SOS1 also has a role in detoxification of ROS as it interacts with RCD1 under salt and oxidative stress (Katiyar-Agarwal et al. 2006). RCD1 is an important transcriptional regulator of oxidative stress responsive genes and has been shown to interact with the C-terminal tail of SOS1, thus clearly emphasizing the involvement of SOS1 in detoxification of reactive oxygen species and prevention of oxidative stress injury (Fig. 2) (Mahajan et al. 2008).

In silico analysis as well as yeast two-hybrid interactions revealed the presence of 25 CIPK genes in the *Arabidopsis* genome and 30 CIPK genes in rice genome (Kolukisaoglu et al. 2004). In contrast to CBL, which are mainly concentrated on chromosomes 4 and 5, the 25 CIPK genes are dispersed among all five *Arabidopsis* chromosomes. In *Arabidopsis* AtCIPK24 is homologous to SOS2. CIPKs do not harbor any decipherable localization signal or any target motif (Kolukisaoglu et al. 2004); therefore, the localization of CIPKs could exclusively be dependent on their respective interaction partner, which would thus serve a dual role as a calcium sensor and as an anchoring protein, regulating the localization and activity of the CIPK at different locations within the cell.

6.1.4 Calcineurin B-Like Proteins

These are a relatively new class of calcium sensors discovered in *Arabidopsis* originally, in the search for the genes imparting salt tolerance and maintaining cellular ion homeostasis (Kudla et al. 1999; Mahajan et al. 2008). Molecular analysis of the salt overlay sensitive mutants opened a new chapter in relation to salt stress signaling that led to the discovery of a pathway that transduces a salt-stress-induced Ca^{2+} signal to reinstate cellular ion homeostasis. The *SOS3* gene, which was identified by Jiang Zhu and colleagues, shares significant sequence homology with *CBL4* from *Arabidopsis* (Mahajan et al. 2006a, 2008). Currently ten CBL and 25 CBLK

genes have been reported from *Arabidopsis* (Mahajan et al. 2006a, 2008). The CBL-CIPK network is also widely distributed among higher plants, but except for *Arabidopsis*, the complexity and characterization of this pathway remains largely unrevealed. As different plants vary in their genome complexity, phenotype, species-specific function or functional diversification can be expected. The essential role imparted by CBL-CIPK genes in stress tolerance necessitates their detailed characterization from higher plants. In fact, there has been no report on experimental characterization of CBL from any higher plant except *Arabidopsis*. *AtCBL3*, in particular has been largely overlooked even in *Arabidopsis*. Recently, we have reported the cloning and characterization of a novel CIPK and its interacting partner CBL from pea. Pea CIPK showed autophosphorylation and could phosphorylate pea CBL. Both pea CBL and pea CIPK were found to be coordinately upregulated in response to various stresses such as cold and salinity but were not coordinately upregulated in response to dehydration stress (Mahajan et al. 2006b).

The localization of a Ca^{2+} -sensor protein to a specific compartment of the plant cell plays an important role in decoding the spatially distinct Ca^{2+} signatures. In silico analysis indicates that some structural features specify subcellular localization for these proteins. The CBL harboring the myristoylation sites, i.e., *AtCBL1*, *AtCBL4*, *AtCBL5*, and *AtCBL9* have been localized predominantly at the plasma membrane (Liu and Zhu 1998; Kim et al. 2000). Moreover, other CBL lacking this myristoylation motif may be primarily cytosolic. This pattern of localization of CBL and their interacting kinases allows specific decoding of Ca^{2+} signatures, which are differentiated spatially within a given cell (Mahajan et al. 2008).

6.2 Without EF-Hand Ca^{2+} -Binding Proteins

There are several proteins that bind Ca^{2+} but do not contain EF-hand motifs. These include the phospholipase D (PLD), annexins, pistil-expressed Ca^{2+} -binding protein (PCP), calreticulin (CRT), calnexin, and forisomes.

6.2.1 Phospholipase D

The activity of PLD, which cleaves membrane phospholipids into a soluble head group and phosphatidic acid, is regulated by $[\text{Ca}^{2+}]_{\text{cyt}}$ through a Ca^{2+} /phospholipids binding site termed the “C2 domain” (Wang 2001). PLD activity is implicated in cellular responses to ethylene and ABA, α amylase synthesis in aleurone cells, stomatal closure, pathogen responses, leaf senescence, and drought tolerance (Wang 2001). Plants possess several PLD isoforms that differ in their affinity for Ca^{2+} and their modulation by phosphoinositides, free fatty acids, and lysolipids (Wang 2001). These biochemical modulators of PLD activity are the substrates or products of phospholipase C, which generates IP_3 , diacylglycerol, phospholipase A_2 , and diacylglycerol kinase, both of which (substrates or products) are regulated by CaM. It is suggested that $[\text{Ca}^{2+}]_{\text{cyt}}$ signaling cascades might coordinate the activities of these diverse enzymes to effect specific responses to the environmental stimuli (Ritchie et al. 2002).

6.2.2 Annexins

Annexins are a family of proteins in plants and animals that bind phospholipids in a Ca^{2+} -dependent manner and contain four to eight repeats of approximately 70 amino acids (Clark and Roux 1995). Annexins are encoded by 12 genes in vertebrates and by eight in higher plants. These proteins are involved in organization and function of biological membranes. Although the exact function of plant annexin is not known, annexins are implicated in secretory processes and some have ATPase peroxidase activities. On the basis of existing literature and experimental evidence, Górecka et al. (2007a) have proposed that plant annexins may have a role in stress response. In fact, Górecka et al. (2007b) have shown that annexin At1 of *Arabidopsis thaliana* (AnnAt1), which is one of eight proteins of this family in *Arabidopsis thaliana*, plays important role in pH-mediated cellular response to environmental stimuli. The same group also suggested that the pH-sensitive ion channel activity of AnnAt1 might play a role in intracellular ion homeostasis.

6.2.3 Pistil-Expressed Ca^{2+} -Binding Protein

A 19-kDa novel PCP expressed in anthers and pistil was isolated (Furuyama and Dzelzkalns 1999). PCP is a high-capacity (binds 20mol of Ca^{2+} per mole of PCP), low-affinity Ca^{2+} -binding protein. PCP has been implicated in pollen-pistil interactions and pollen development.

6.2.4 Calreticulin

CRT is an abundant Ca^{2+} -binding protein (Ca^{2+} sensor) and was first detected in the ER of rabbit skeletal muscle (Ostwald and MacLennan 1974). CRT plays a crucial role in many cellular processes, including Ca^{2+} storage and release, protein synthesis, and molecular chaperone activity. Besides its main location in the ER (Opas et al. 1996), CRT has been found to reside in the nuclear envelope (Napier et al. 1995), the spindle apparatus of the dividing cells (Denecke et al. 1995), the plasmodesmata in the root apex (Baluska et al. 1999), and on the cell surface (Gardai et al. 2007), indicating that CRT is essential for normal cell function. In plants it was first detected and isolated from spinach leaves (Menegazzi et al. 1993). Later, cDNA clones of CRT were isolated from *Arabidopsis* (Huang et al. 1993), barley (Chen et al. 1994), maize (Napier et al. 1995), tobacco (Denecke et al. 1995), pea (Hassan et al. 1995), *Brassica rapa* (Lim et al. 1996), *Ricinus communis* L. (Coughlan et al. 1997), and rice (Li and Komatsu 2000). Plant CRT shares the same structural domain features and basic functions identified for animal CRTs; therefore, the plant CRT might also be involved in regulation of Ca^{2+} homeostasis and Ca^{2+} -dependent signal pathways (Wyatt et al. 2002). Plant CRT is highly expressed during mitosis in tobacco (Denecke et al. 1995), embryogenesis of barley (Chen et al. 1994), *Nicotiana plumbaginifolia* (Borisjuk et al. 1998), and maize

(Dresselhaus et al. 1996), and in flower tissues, including pollen tubes as well as anthers (Nardi et al. 2006). Increasing evidence also indicates that this protein is involved in the plant response to a variety of stress-mediated stimuli. Recently, wheat CRT (TaCRT) was shown to be involved in the plant response to drought stress, indicating a potential in the transgenic improvements of plant water-stress (Jia et al. 2008).

6.2.5 Calnexin

Calnexin (CNX) is one of the important ubiquitous calcium-binding proteins, and was initially identified as an ER type I integral membrane protein (Sarwat and Tuteja 2007). It acts as a chaperone that share several functions, including Ca^{2+} binding, lectin-like activity, and recognition of misfolded proteins. CNX binds to monoglucosylated carbohydrate on newly synthesized glycoproteins. It consists of a large (50-kDa) N-terminal calcium-binding luminal domain, a single transmembrane helix, and a short (90-residue), acidic cytoplasmic tail. CNX together with its teammates, such as ERp57 (a protein disulfide isomerase like protein resident in the ER), and CRT comprise the so-called CNX/CRT cycle which is responsible for the correct folding of newly synthesized proteins and glycoproteins before their translocation to the secretory pathway (Sarwat and Tuteja 2007). Huang et al. (1993) isolated a cDNA encoding a CNX homologue CNX1p from *Arabidopsis thaliana*, a transmembrane protein of type I topology, and it showed 48% identity with dog CNX. Sequences encoding CNX homologues have also been cloned from maize (Kwiatkowski et al. 1995), soybean (Goode et al. 1995), and *Pisum sativum* (Ehtesham et al. 1999). Li et al. (1998) demonstrated the possible role of CNX in folding and assembly of vacuolar H^+ -ATPase from oat seedlings along with another ER chaperone, BiP. Pea CNX was shown to be a constitutively expressed 72.5-kDa phosphoprotein. The acidic domain of CNX at the N-terminus present in the ER lumen may be involved in Ca^{2+} binding. *Arabidopsis thaliana* CNX lacks both of the acidic domains; hence, it is unlikely to be involved in low-affinity Ca^{2+} binding. Similarly, pea CNX also lacks the C-terminal acidic domain, but possesses a potential Ca^{2+} -binding domain at the N-terminus (Ehtesham et al. 1999).

6.2.6 Forisomes

Forisomes are giant contractile motor protein bodies specific for the sieve tubes of Fabaceae plants (legume) and function as a gatekeeper in phloem sieve tubes. They are unique protein bodies which act as cellular stopcocks, by undergoing a Ca^{2+} -dependent and ATP-independent conformational switch in which they plug the sieve element (Eckardt 2001; Knoblauch et al. 2001). In living cells, this reaction is probably controlled by Ca^{2+} transporters in the cell membrane. Knoblauch et al. (2003) showed that an influx of calcium into legume sieve elements stimulates the rapid and reversible dispersal of crystalloid P-protein aggregates to occlude sieve

plate pores, which may be produced by wounding or pathogen-mediated mechanical injury. Overall, this helps the plant to stop hemorrhaging nutrients and to prevent entry of pathogens; because of this property the name “forisome” has been given to the proteins (Latin *foris* meaning “the wing of a gate,” Greek *soma* meaning “body”) (Knoblauch et al. 2003). Forisomes also control flux rates in the phloem of faboid legumes by reversibly plugging the sieve tubes. These proteins undergo an anisotropic shape transition (longitudinally expanded to contracted) in response to ion concentration changes (Ca^{2+} , H^+ , etc.). Forisomes were mostly located close to sieve plates, and occasionally were observed drifting unrestrainedly along the sieve element, suggesting that they might be utilized as internal markers of flow direction. Recently, forisome protein aggregates received broader attention owing to their ability to convert chemical into mechanical energy. The *for1* gene is highly conserved among Fabaceae species and appears to be unique to this phylogenetic lineage since no orthologous genes have been found in other plants, including *Arabidopsis* and rice (Noll et al. 2007). It is hypothesized that forisome plugs are removed once the cytosolic calcium level has returned to the initial level in those sieve tubes (Furch et al. 2007). Peters et al. (2007) isolated tailed forisomes of the sword bean *Canavalia gladiata* and reported that Ca^{2+} induced a sixfold volume increase within about 10–15 s; the reverse reaction following Ca^{2+} depletion proceeded in a fraction of that time. They suggested that *Canavalia gladiata* provides a superior experimental system, which will prove indispensable in physiological, biophysical, ultrastructural, and molecular studies on the unique ATP-independent contractility of forisomes.

7 Conclusions

Evolution has adopted positively charged calcium as the primary signaling element of cells. Ca^{2+} is one of the principal second messengers for functioning as a central node in the overall “signaling web” and plays an important role in providing stress tolerance to plants. Analysis of $[\text{Ca}^{2+}]_i$ dynamics has demonstrated its signaling role in plant cells in response to a wide array of environmental cues. In general, the stress leads to increased cytosolic Ca^{2+} , which initiates the stress signal transduction pathways for the stress tolerance. Ca^{2+} is the most tightly regulated ion within all membrane-bound organisms and binds to several proteins to effect changes in localization, association, and function. It is now clear that Ca^{2+} signaling affects almost every aspect of the cellular metabolism of living organisms. In fact, an indirect increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in plant cells is mediated by the increase in intracellular concentrations of other second messengers such as cyclic GMP, cyclic AMP, and IP_3 , in response to stimuli. The elevation of Ca^{2+} concentration in cytoplasm is found to be a key event in the plant cell for transduction of various signals to a biological effect. This suggests that Ca^{2+} is a communication point in the cytoplasm for the cross talk between different signal transduction pathways and finally helps in the interchanging of the information. The Ca^{2+} signaling involves many sensor proteins

that decode temporal and spatial changes in cellular Ca^{2+} concentration. Cells usually invest much of their energy to effect changes in Ca^{2+} concentration. Recently, in yeast it has been reported that there is cross talk between Ca^{2+} -calcineurin signaling, nutrient sensing, and regulation of the cell cycle (Zhang and Rao 2008). Overall, the involvement of Ca^{2+} in various metabolic processes in plant might have general implications.

Acknowledgements I thank Renu Tuteja for critical reading of and corrections to the article. This work was partially supported by the grant from the Department of Biotechnology (DBT), Government of India. I apologize to the many scientists whose work I was not able to credit owing to space restrictions. In most cases, reviews have been cited at the expense of the original work.

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Signaling in Plants

Baluška, F.; Mancuso, S. (Eds.)

2009, XII, 308 p. 22 illus., 2 illus. in color., Hardcover

ISBN: 978-3-540-89227-4