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Calcium Signaling: Linking Environmental Signals to Cellular Functions

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I. INTRODUCTION

Calcium ions are ubiquitous signal molecules. In plants, a number of external stimuli lead to changes in cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$), which in turn regulate a wide variety of responses (see Bush, 1995). Besides, several physiological processes are also accompanied with changes in $[Ca^{2+}]_{cyt}$ (Trewavas and Malho, 1997, 1998). Both these facts emphasize the role of calcium ions as one of the most important messengers involved in signal-response coupling.

Regulation of calcium level is important for the survival of the cell. The cytosolic Ca^{2+} concentration is delicately balanced by the presence of a large number of calcium stores like vacuoles which can even release calcium when required, specific channels/pumps that regulate the movement of Ca^{2+} in and out of cells and organelles, and different (calcium-binding) proteins that bind to calcium either to sequestrate it or to perform some other complex tasks. All these components involved in regulation of calcium concentrations in its equilibrium level constitute the complex network of calcium homeostasis system.

The role of calcium ion as an important signal molecule and the various components involved in maintaining calcium homeostasis and calcium signal transduction have been reviewed previously by many workers (Bowler and Chua, 1994; Bootmann and Berridge, 1995; Bush, 1995; Webb et al., 1996; Berridge, 1997; Trewavas and Malho, 1997; McAinsh and Hetherington, 1998; Trewavas, 1999). Despite intense activity in this area of research, one of the main questions that has eluded a clear answer is how calcium operates as a signal molecule in response to various extracellular factors with precision and a high degree of specificity. With the availability of newer sophisticated techniques such as Ca^{2+} fluorescence measurements using apoaequorin, Ca^{2+} ratio imaging, confocal microscopy, flow cytometry, introduction of indicator dyes and other compounds directly in the cells, together with molecular biological approaches, it has now become possible to study the nature of specific "calcium signatures" involved with a specific response (Knight and Knight, 1995). In this review we have focussed primarily on collecting information and its implications related to an understanding of how speci-

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ficity is achieved via calcium signal transduction pathway.

II. CALCIUM IN SIGNAL-RESPONSE COUPLING

A. External Stimuli Causing the Change in Calcium Levels

Different external stimuli induce a small, localized change in the level of Ca^{2+}, which drastically alter the cytosolic gradients thus finally leading to a change in the Ca^{2+} homeostasis of the cell. This change or disturbance of Ca^{2+} level is perceived by the cellular machinery as a signal. External signals such as light, hormones, and stress involve a change in cytoplasmic calcium level, that could differ in source, pattern of change, or duration of change.

1. Stress

Plants withstand various stress conditions by responses mediated via changes in cytoplasmic calcium concentration. Cold shock responses have been studied in the case of maize suspension culture cells (Campbell et al., 1996) and *Arabidopsis* seedlings (Knight et al., 1996), where a biphasic pattern consisting of fast transient and slow prolonged increase in calcium concentration was observed. Similarly, using cold sensitive tobacco and cold resistant *Arabidopsis* seedlings, it has been shown that the plants seem to have a cold calcium memory and a plant-specific calcium signature in response to cold acclimation. In cold signaling, the source of calcium could be either extracellular or intracellular, and accordingly the responses could vary from a fast transient increase to complex wave-like patterns (Monroy and Dhindsa, 1995). Recently, heat shock-induced changes in Ca^{2+} level have been shown to be correlated with thermotolerance in tobacco seedlings (Gong et al., 1998).

The response of plants to hypoxia has been studied in *Arabidopsis* seedlings where a biphasic pattern of calcium change was observed. Calcium channel inhibitors differentially blocked the two phases of the response, thus showing involvement of different calcium sources (Sedbrook et al., 1996). Similarly, water stress in ageotropic pea mutants (Takano et al., 1997) and senescence in detached parsley leaves (Huang et al., 1997) has also been reported to involve a change in cytoplasmic calcium levels. Hypoosmotic shock to the tobacco cells has been shown to induce a biphasic cytosolic response, where the first transient increase is mediated by external stores, followed by the second transient increase from the internal calcium stores (Cessna et al., 1998).

In *Arabidopsis* seedlings, response to drought and salinity stress has been shown to be mediated via changes in calcium level. A transient elevation of calcium was observed that could be blocked substantially by EGTA or lanthanum. Besides, expression of three genes, *p5cs, rab18,* and *Iti78,* was induced by salt or mannitol treatment. Stimulation of all these genes by mannitol could be inhibited by pretreatment with lanthanum, but in the case of salt-stimulated expression lanthanum could inhibit the expression of *p5cs* only. Mannitol induction of *p5cs* was also blocked by EGTA, gadolinium, and verapamil, further confirming the role of calcium in this response. Involvement of IP_3 released calcium from vacuoles has also been shown in this case (Knight et al., 1997). Role of calcium in plant salt tolerance has also been shown by Liu and Zhu (1998) where they have shown the involvement of a calcium sensor that has similarity to calcineurin. Besides a salt-induced gene cloned from *Arabidopsis* (AtCP1) has turned out to be a calcium binding protein (Jang et al., 1998), giving more evidence for involvement of calcium in salinity responses.

Elicitor-induced signaling has also been shown to be mediated via calcium ions as shown by Gelli and Blumwald (1997) using tomato protoplasts. Similarly, oligonucleoside-elicitor mediated signaling is also mediated via calcium ions (Ebel, 1998). Besides, recently it has been shown that ozone-induced signaling is also mediated by a transient increase in cytosolic calcium level (Clayton et al., 1999).

2. Mechanical Signals

Mechanical signals such as touch, wind, and gravity have long been shown to be mediated via a change in cytoplasmic calcium concentration (Bjorkman and Cleland, 1991; Halley et al., 1995). In transgenic plants expressing aequorin it has been observed that in response to different signals various calcium spikes or waves are produced that are specific to a particular signal (Knight et al., 1997).

3. Hormones

Involvement of calcium has also been shown in various responses mediated via hormones (Bush et al., 1992, 1993). Changes in calcium levels vary from transient increase as in case of *S. Alba* in response to auxin, to ramping pattern as in case of maize in response to auxin (Gehring et al., 1990). The effect of GA on barley and wheat aleurone protoplasts have been very well connected to changes in calcium levels (Johannes et al., 1992; Gilroy, 1996; Kuo Anling et al., 1996). Similarly, ABA and cytokinins have also been shown to bring about a change in calcium levels (Sanders and Hepler, 1994; Gilroy, 1996; Staxen et al., 1999).

4. Light

Light-mediated responses have been shown to be accompanied with changes in cytoplasmic calcium concentration (Tretyn et al., 1991; Chory, 1994, 1997). Red light-mediated responses have been studied in oat protoplasts, maize coleoptiles, and mesophyll protoplast of wheat, and a fast transient increase in calcium levels have been observed (Das and Sopory, 1985; Chae et al., 1990; Shacklock et al., 1992; Mehta et al., 1993). Similarly, blue light also causes a change in calcium concentration. In *Chlamydomonas* system *gsa* gene that encodes for early chlorophyll biosynthetic enzyme, is specifically induced by blue light in the presence of acetate, whereas no stimulation could be detected in the absence of acetate. Calcium could partially supplement for acetate requirement, although the level is low. Both calcium channel blockers and calmodulin antagonists affect the induction of *gsa* gene, suggesting calcium and calmodulin are involved in signal transduction pathway linking the blue light perception and induction of gene (Im et al., 1996). In guard cells verapamil inhibits the stomatal blue light responses, giving further clues about the involvement of calcium channels in blue light-mediated responses (Shimazaki et al., 1997).

Involvement of calcium has also been shown in plant-pathogen interaction by modulating the expression of virulence genes of the pathogen (Flego et al., 1997) and cell to cell communication (Trucker and Boss, 1996). Besides, Ca^{2+} also mediates primary leaf development in *Sorghum bicolor*, which is a phytochrome-dependent phenomenon. Exogenous calcium at 3 to 5 m*M* concentration can replace the light requirement for the formation of primary leaves in dark grown plants (Sanan and Sopory, 1998).

B. Physiological Responses Caused by Altered Calcium Levels

Besides external stimuli affecting the calcium levels, some physiological responses are also preceded with the changes in the calcium level. The physiological responses are generally very complex in nature. However, in some instances very specific spatial and temporal patterns of calcium changes have been observed, for example, during growth and reorientation of pollen tubes, nodulation, and during stomatal movements. In most of these cases the pattern is biphasic, where a small and fast transient increase in calcium levels is followed by large and complex pattern of oscillations and waves. The visualization of these complex patterns of calcium changes has been possible because of two recent advances made in this field, viz., introduction of indicator dyes in the living plant cells and the ability to introduce molecules that can directly alter calcium levels. Both these methods coupled to the use of confocal microscopy have given direct evidences for the complex changes taking place in the cells during various physiological responses. In the case of *Commelina* guard cells, both symmetrical

and asymmetrical oscillations were observed depending on the strength of external calcium concentration (McAinsh et al., 1995), while in the case of pollen tube growth and reorientation, a slow Ca^{2+} wave was observed, moving toward the tip of the growing pollen tube (Franklin-Tong et al., 1996; Malho and Trewavas, 1996). This reorientation is correlated with the relocation of a calcium-dependent kinase activity (Moutinho et al., 1998). Similarly, in growing root hairs also a tip focused gradient of calcium was observed that moves like a wave (Bibikova et al., 1997). A wave-like pattern of calcium change has also been observed in case of *Fucus* rhizoids (Taylor et al., 1996). All these examples suggest that possibly all the apically growing cells have a similar mechanism of calcium change, although it might not be the only factor (Malho, 1998).

Another very interesting pattern of calcium change was observed in the case of nodulation in alfalfa roots with specific *Rhizobium* strain. Asymmetric spikes of calcium are produced that start at the nucleus of the cells and move outward as a slow-moving wave, that is, in this process both an oscillatory as well as a wave-like pattern of calcium change could be observed (Ehrhardt et al., 1996). Circadian responses in plants are also mediated by the regulation of calcium fluxes in plants (Johnson et al., 1995). Similarly, pollination and fertilization in higher plants have been shown to be mediated by intracellular calcium fluxes (Wilhelmi and Preuss, 1999). A description of various processes accompanied with $[Ca^{2+}]_{cyt}$ is given in Table 1 showing that changes in $[Ca^{2+}]_{cyt}$ is central to the theme of cellular signaling.

C. Gene Expression Directly Involving Altered Calcium Level

The regulation of gene expression by calcium could be modulated via a variety of Ca^{2+}-binding proteins, CaM-binding proteins, several kinases, and phosphatases. In animal systems, Ca^{2+} has been shown to effect expression of several genes at both transcriptional and translational levels (see Hunter and Karin, 1992; Finkbeiner and Greenberg, 1997; Santella and Carafoli, 1997).

In plants, there are a few reports of Ca^{2+}-regulated gene expression (Table 2). Direct regulation of gene expression by calcium has been shown in the case of ethylene-dependent responses (Raz and Flurh, 1992). Ethylene is one of the inducers of a plant pathogenesis-related gene, chitinase. By blocking calcium fluxes with chelators as EGTA, ethylene-dependent accumulation of chitinase could be specifically blocked, whereas chitinase accumulation could be increased by artificially increasing the $[Ca^{2+}]_{cyt}$ with ionomycin or thapsigargin.

Expression of two annoxia-inducible genes was also shown to be regulated by changes in cytosolic calcium concentrations (Subbaiah et al., in 1994). Maize suspension culture cells responded to annoxia by an increase in $[Ca^{2+}]_{cyt}$ level, and the source of this calcium was intracellular. Expression of *adh1* and *sh1* genes that are directly related to annoxia responses was preceded with changes in $[Ca^{2+}]_{cyt}$, and the induction of both the genes was blocked by ruthenium red, a calcium channel blocker. Similar effects were obtained with verapamil and bepridil that failed to block the $[Ca^{2+}]_{cyt}$ rise as well as anoxic gene expression, whereas caffeine that induced $[Ca^{2+}]_{cyt}$ could also induce *adh1* gene expression. Involvement of calcium has also been shown in stress-regulated gene expression in protoplasts incubated in Ca^{2+} solutions and given various stimuli (Sheen, 1996).

Calcium has been shown to effect the expression of cold acclimation-specific genes. Calcium is an important messenger in low-temperature signaling and a correlation between calcium availability, *cas* (*c*old *a*cclimation *s*pecific), gene expression and freezing tolerance has been shown (Monroy et al., 1993). Calcium chelator and calcium channel blockers inhibited the calcium influx as well as the expression of *cas15* and *cas18* genes at 4°C, whereas calcium ionophore or calcium channel agonist induced the *cas15* and *cas18* gene expression as well as calcium influx (Monroy and Dhindsa, 1995). Expression of another cold-induced gene *kin* was also inhibited by EGTA, La^{3+}, and Gd^{3+}. Ruthenium red, an inhibitor of calcium release from intracellular stores, partially inhibited cold tolerance as well as *kin* gene expression (Tahtiharju et al., 1997). Similarly, ex-

TABLE 1
Physiological Responses Involving Changes in $[Ca^{2+}]_{cyt}$ Level

Processes	System	Pattern	Source of Ca^{2+}	Ref.
Stomatal opening	*Commelina* guard cells	Symmetrical or asymmetrical oscillations dependent on strength of signal	Extracellular and partially intracellular	McAinsh et al., 1995
Pollen tube growth and reorientation	*Papver roheas* *Agapanthus umbellatus*	SV channels, a slow-moving Ca^{2+} wave is generated reaching toward growing pollen tube tip	Intracellular and extracellular, IP_3 mediated influx and clustering of active channel at the tip	Franklin-Tong et al., 1996 Malho et al., 1995 Malho and Trewavas, 1996
Pollen-mediated self-incompatibility	*Papver roheas*	Localized Ca^{2+} transient	IP_3 mediated release from intracellular stores	Franklin-Tong et al., 1993, 1995
Cell volume control	*Fucus* rhizoids	Transient increase in apical region that spreads like a wave in sub-apical region	Initial increase by extracellular stores, wave-like pattern by intracellular stores	Taylor et al., 1996
Nodulation	*Alfalfa* roots and specific *rhizobium*	Asymmetric spikes of Ca^{2+}, starts at nucleus and travels outward as a slow-moving wave	Initial spikes by internal stores, extracellular stores involved in sustained increase	Ehrhardt et al., 1996
Fertilization	Maize *in vitro* fertilization system	Transient increase		Digonnet et al., 1997

TABLE 2
Calcium-Regulated Gene Expression in Plants

System	Gene (s)	Regulation	Ref.
Ethylene-related pathogenesis response in tobacco	Chitinase	Up-regulation by ethylene, Ca^{2+}, ionomycine, thapsigargin Down-regulation by EGTA	Raz and Fluhr, 1992
Annoxia response in maize suspension culture cells	*adh*1, *sh*1	Down-regulated by Ruthenium red, Verapamil and Bepridil	Subbaiah et al., 1994
Sugar inducible gene expression in sweet potato and transgenic tobacco	β-amylase Sporamine	Up-regulation by metbolizable sugars, PGA Down-regulation by EGTA, CaM inhibitors, La^{3+}, Diltiazem, Nicardipine, Verapamil	Ohto et al., 1995
Cold acclimation in Alfalfa cell suspension culture	*caa* 15, *cas*18	Up-regulation by A23187, Bay K 8644 Down-regulation by BAPTA, La^{3+}, Nitrendipine, Verapamil	Monroy and Dhindsa, 1995
Cold acclimation in *Arabidopsis thaliana*	*kin*	Down-regulation by EGTA, La^{3+}, Gd^{3+} and partially by ruthenium red	Tahtiharju et al., 1997
Cold shock in transgenic tobacco	THC 2, TCH 3, and TCH 4	Down-regulation by La^{3+}, Gd^{3+}, and BAPTA	Polisensky and Braam, 1996
UV-B/UV-A induced expression in Arabidopsis cell suspension culture	*chs*	Down-regulation by nifedipine, ruthenium red, W7, K252a, staurosporine, okadaic acid, and cycloheximide	Christie and Jenkins, 1996
Phytochrome-mediated responses	*cab, rbc*S, LHCII, OEEI, D1, D2, α, γ, subunit of ATP synthase	Up-regulation by Ca^{2+}, Ca^{2+}-activated CaM	Neuhaus et al., 1993, 1997 Bowler et al., 1994 Mustilli and Bowler, 1997
Light-mediated response in maize	*cab-m1*	Up-regulation by Ca^{2+} Down-regulation by EGTA, W7	Shiina et al., 1997
ABA signaling through *cADP-R* in *aurea* mutant of tomato	*rd–29A, kin2*	Up-regulation of ABA, Ca^{2+}, *cADP-R,* ADP-ribosyl cyclase, okadaic acid Down-regulation by EGTA, NADase, staurosporine , K252a	Wu et al., 1997

pression of TCH genes of *Arabidopsis* in response to cold shock is directly regulated by changes in intracellular calcium levels (Polisensky and Braam, 1996). Transgenic *Arabidopsis* plants expressing apoaequorin showed that source of this calcium is extracellular. Expression of TCH2, TCH3, and TCH4 genes was down-regulated by La^{3+}, Gd^{3+}, and BAPTA, all of which are blockers of plasma membrane calcium channels.

ABA induces expression of two genes in *aurea* mutants of tomato, *rd–29A*, a desiccation-induced gene and *kin2*, a cold-responsive gene. Ca^{2+} or cyclic ADP ribose (cADP-R), an endogenous regulator of calcium could replace the ABA requirement for the induction of these genes, while EGTA could inhibit the induction. ADP ribosyl cyclase, which synthesizes cADP-R also induced the expression, whereas NADase, which degrades NAD^{+}, a precursor of cADP-R and 8 NH_2–cADP-R, an inactive analogue of cADP-R inhibited the expression. Involvement of kinases and phosphatases has also been shown in expression of these genes as kinase inhibitors, staurosporine and K252a, inhibited the induction of the genes and okadaic acid induced the expression (Wu et al., 1997). Involvement of Ca^{2+} and CaM in regulation of gene expression in phytochrome-mediated responses has also been shown (Neuhaus et al., 1993; Bowler et al., 1994; Wu et al., 1996; Mustilli and Bowler, 1997; Neuhaus et al., 1997). In the case of *aurea* mutants of tomato, Ca^{2+} was shown to regulate the expression of various genes. By microinjecting the single cells, it was observed that Ca^{2+} and CaM directly effect expression of cab-GUS reporter gene. Besides, expression of some other genes such as *RbcS*, LHCII, OEE1, D1, D2 (from PSII), and α and γ subunit of ATP synthase was also regulated by Ca^{2+} and/or CaM. This fact also showed that Ca^{2+} could control expression of nuclear genes as well as of chloroplast genes. As synthesis of chloroplast-encoded proteins is dependent on transcriptional, posttranscriptional, and translational regulatory mechanisms, it also shows the astonishing effectiveness of Ca^{2+} in being able to modulate cellular processes in plants at several fundamental levels. Similar results were obtained using Ca^{2+} and CaM along with cGMP (Bowler et al., 1994), where expression of $Cytb_6f$, RUBISCO, and genes of PS I (photosystem I) was directly regulated. These preliminary experiments suggested that phytochrome-regulated responses could be grouped into three classes, viz., Ca^{2+}/CaM-regulated responses, for example, expression of *cab* gene (Neuhaus et al., 1993), cGMP-regulated responses, for example, *chs* gene expression (Bowler et al., 1994), and responses regulated by both Ca^{2+}/CaM and cGMP, for example, expression of *fnr* and *as1* gene (Bowler et al., 1994; Neuhaus et al., 1997). Using *rbcS–3A* and *chs* promoters, two small *cis*-acting elements have been identified. Box II, which is present at –90 to 35 S promoter, is a target for calcium-mediated pathway and Unit I is a target for cGMP pathway (Wu et al., 1996). Both these *cis* elements also serve as a target for reciprocal control mechanisms and are affected by cGMP as well as Ca^{2+}. Similarly, a 17–bp *cis* element is necessary and sufficient for *as1* gene regulation with Ca^{2+} and cGMP. Thus, it shows that calcium is also involved in cross-talk between different pathways. Our work on *PsaF* gene (encoding subunit III of photosystem I reaction center) also show that there may be calcium-responsive elements in the upstream promoter region that is involved in the light-mediated expression of this gene (Chandok, Sopory, and Oelmueller, submitted).

Calcium is involved in both UV-B and UV-A/blue light photo-transduction pathway regulating *chs* gene expression (Christie and Jenkins, 1996). Calcium channel blockers nifedipine and ruthenium red inhibited the induction, whereas La^{3+} and verapamil had no effect, indicating the involvement of intracellular calcium stores. CaM inhibitor such as W7, kinase inhibitors such as staurosporine and K252a, and phosphatase inhibitor okadaic acid inhibited the induction of this gene showing the possible involvement of kinases and phosphatases in this process. The induction also involved the synthesis of new proteins such as cycloheximide could inhibit the expression. Recently, Shiina et al. (1997) have shown that in maize expression of *cab-m1* gene is regulated specifically by a light-induced increase in $[Ca^{2+}]_{cyt.}$ This increase is blocked by EGTA and W7, a CaM inhibitor, suggesting an involvement of Ca^{2+} as well as CaM. Analysis of promoter elements showed that a short (54 bp) region in the

promoter (–953 to –899) is responsible for calcium responsiveness.

Another interesting example of Ca^{2+}-induced gene expression is in sugar inducible expression of genes coding for sporamine and β-amylase of sweet potato (Ohto et al., 1995). Both these genes are up-regulated by high levels of metabolizable sugars and polygalacturonic acid. CaM inhibitors and EGTA could inhibit the expression of both these genes in cut petioles as well as in transgenic tobacco plants expressing a fusion gene having β-amylase promoter and *GUS* reporter gene.

Calcium channel blockers La^{3+}, diltiazem, nicardipine, and verapamil also showed similar effects on the expression of a β-amylase-*GUS* fusion gene. As metabolizable sugars also increase $[Ca^{2+}]_{cyt}$, it might be involved in cross-talk between carbohydrate metabolism and other stimuli. To summarize, it follows that diverse signals mediate their response through a single ion, that is, Ca^{2+} and this small dynamic molecule by interacting with a large number of proteins and kinases seems to regulate expression of various genes, thereby controlling many cellular processes.

III. SPECIFICITY OF Ca^{2+} SIGNALS

As many diverse stimuli cause a change in the level of Ca^{2+}, an important question that arises is how calcium transduce a specific signal for so many diversified responses? The answer probably lies in the extensive network of proteins, factors, pumps, channels, and stores, etc., which delicately balance the Ca^{2+} homeostasis and the nature of proteins with which Ca^{2+} interacts.

The specificity of Ca^{2+} signal is obtained probably at three different levels. The cell type and its physiological conditions, specific spatial and temporal patterns of $[Ca^{2+}]_{cyt}$ changes in response to a specific stimulus, and changes in concentration or activity of proteins like kinases, phosphatases, and other calcium binding proteins in concert with changes in $[Ca^{2+}]_{cyt}$ levels.

A. Cell Type and Physiological Conditions of the Cell

The initial control of specificity in calcium signaling could be obtained at the level of cell type. Under natural environmental conditions plants are simultaneously exposed to a variety of stimuli and accordingly react to all of them in an orchestrated manner. However, not all the signals are perceived and responded to by all the cells. To perceive a signal or stimulus, only some specific cells would have the receptors. Besides, even if cells perceive the stimulus they might not have the machinery to respond to a particular stimulus. Thus only those cells that express the genes required to code for all the constituent of the signaling pathway would respond to a particular signal. Expression of these genes is again subjected to the control by the local environment of the cell and growth and developmental conditions of the cell. All these factors may decide the specificity of the calcium signaling at the preliminary level. Besides, sometimes same stimulus can cause opposite overall change in Ca^{2+} level, depending on the cell type. For example, ABA causes a decrease in cytoplasmic Ca^{2+} in case of barley aleurone protoplast, while in other cell types, such as stomatal guard cells in *Vicia faba* (Schroeder and Hagiwara, 1990) or *Commelina* (Gilroy et al., 1991), ABA increases the level of Ca^{2+}.

B. Source of Ca^{2+}

In many instances it has been demonstrated that the source of Ca^{2+} in response to a particular stimuli could be different and thereby determine the specificity of a response. This was shown by the use of different channel inhibitors that specifically block influx channels, for example, La^{3+} blocks PM channels (Felle and Hepler, 1997), Gd^{3+} blocks stretch-activated channels (Malho and Trewavas, 1996), ruthenium red is an inhibitor of ER and mitochondrial calcium channels (Knight et al., 1992), and compounds such as neomycin and heparin inhibit PLC activity, thus inhibiting the production of IP_3 or mastoparan that stimulates IP_3 production (Franklin-Tong et al., 1996).

In response to stimuli such as elicitor-induced changes in tomato protoplasts (Gelli and Blumwald, 1997), GA-induced changes in barley aleurone protoplasts (Gilroy, 1996), and water stress in ageotropic pea mutants (Takano et al., 1997), it was shown that the source of calcium is

extracellular or apoplastic, which by influx through plasma membrane channels causes elevation of $[Ca^{2+}]_{cyt}$. In some other cases $[Ca^{2+}]_{cyt}$ elevated levels were due to release from intracellular stores, for example, in wind- and touch-induced changes (Knight et al., 1992), cytokinin-mediated responses (Schumaker et al., 1993), and ABA-mediated responses (Gilroy, 1996). However, sometimes the distinction between different sources is not so clear, and an involvement of both extracellular and intracellular calcium has been shown, for example, in case of cold shock-induced changes (Knight et al., 1996). Cold shock first causes a large transient increase in $[Ca^{2+}]_{cyt}$, which is due to influx from extracellular stores. This is followed by small changes observed in vacuolar micro-domain that are prolonged and show an involvement of IP_3-mediated release from intracellular stores. Prior exposure to cold or H_2O_2 causing acclimation of plants to cold chilling sensitive (tobacco) and chilling resistant (*Arabidopsis*) plants resulted in a modification in the Ca^{2+}-related changes, showing that calcium has a specific signature in different plants (Knight et al., 1996; Monroy and Dhindsa, 1995).

In the case of maize suspension culture cells, hypoxia-induced calcium changes are of intracellular origin only, whereas in *Arabidopsis* calcium-mediated response to hypoxia shows a biphasic pattern (Sedbrook et al., 1996). A rapid transient increase was observed first that was followed by a slow prolonged response that lasted for 1.5 to 4 h. Calcium channel blockers affect these responses differentially, suggesting their different origin. This again suggests that different cell types might mediate the response through different stores in response to a similar signal.

Also, in the case of guard cells vacuoles a biphasic pattern of calcium change was obtained, and it has been attributed to the presence of slow voltage-activated channels (Ward and Schroeder, 1994). These channels get activated by increase in $[Ca^{2+}]_{cyt}$, showing the phenomenon of calcium-induced calcium release (CICR), which is very common in animal systems (Berridge, 1995). CICR could also help to understand the process of biphasic responses in other systems.

In detached parsley leaves, leaf senescence has been shown to be correlated with the increase in cytosolic calcium, but in this particular case the increase is not because of Ca^{2+} influx or release from intracellular stores but due to inability of Ca^{2+} efflux transport system to remove extra calcium (Huang et al., 1997). Thus, all these processes show that the source of elevated calcium itself is very different for different stimuli, which could provide the needed specificity.

C. Spatial and Temporal Patterns of Ca^{2+} Changes

One of the most crucial properties of calcium, which gives specificity to the calcium signaling pathways, is its low diffusibility. Because of existence, affinity, and specificity of a large number of Ca^{2+} binding proteins, Ca^{2+} can migrate no more than 0.1 to 0.5 μm lasting only –50 μs in the cytoplasm, although the predicted value from the ionic radii is 1000 $\mu m^2/s$ (Clapham, 1995). Therefore, Ca^{2+} changes are extremely localized. These localized changes could have many specific consequences, such as in response to a particular kind of stimulus, the level of Ca^{2+} could change in the close proximity of a particular Ca^{2+} store. In other words, for different kinds of stimuli, different Ca^{2+} sources could be involved. Besides, a localized high level of Ca^{2+} at a particular area in cytosol will affect specific proteins or second messengers in that area only. This low diffusibility could also provide specific patterns of calcium change as a spike or a wave or ramping pattern, symmetrical or asymmetrical oscillations, etc. Thus, the control on specificity of calcium signal could be obtained at various levels of pathway.

Spatial and temporal patterns of Ca^{2+} changes are very different for different stimuli providing another dimension to the specificity of Ca^{2+} signal (Thomas et al., 1996). In response to some stimuli, Ca^{2+} changes are transient, for example, in case of wheat mesophyll responses to red light (Shacklock et al., 1991), in maize *in vitro* fertilization (Digonnet et al., 1997), or cytokinin responses to *Paphiopedilium* guard cells (Johannes et al., 1992). In some other cases, a steady state increase is observed, for example, in the case of cytokinin responses to *Funaria* caulonema (Hahm and Saunders, 1991). Thus, cytokinin causes a rapid

transient increase in *Paphiopedilium* and a steady state increase in *Funaria*. Similarly, GA causes a rapid steady state increase in wheat aleurone protoplasts, and a slow steady-state increase in barley aleurone protoplasts (see Bush, 1995), showing that the pattern of the change also depends on the cell type responding to a particular signal. In some other cases the spatio-temporal patterns of Ca^{2+} changes are much more complex. Two such novel patterns are calcium waves, which provide a spatial heterogeneity to the calcium signal and calcium oscillations, which provide the temporal heterogeneity.

1. Calcium Wave

Calcium wave is a change in concentration of calcium starting from a single localized origin that slowly moves throughout the cell, that is, concentration change starts at one point and then radially disseminate to adjoining areas, finally covering the whole cell. As shown in animal systems, a calcium wave could be involved in transmitting a biochemical information from a localized source to the whole cell or tissue (Tsien and Tsien, 1990; Jeffe et al., 1993).

In plants, the existence of a wave-like pattern of calcium change has been reviewed recently (Trewavas, 1999). It is best studied in the case of growth and orientation of pollen tubes and cell volume control in *Fucus* rhizoids. During growth and orientation of pollen tube in case of *Agapanthus umbellatus* (Malho et al., 1995; Malho and Trewavas, 1996), and *Papaver rhoeas* (Franklin-Tong et al., 1996) it has been observed that clustering of active ion channels in the tip area leads to the formation of a tip focused gradient of calcium. This indicates that the role of ion channels is central to the mechanism that ensures successful fertilization, which in turn depends on the correct orientation of the pollen tube and micropyle. This gradient of calcium leads to the origin of a calcium wave starting at the sub-apical regions of the pollen tube and migrating toward the tip. As soon as the wave reaches the tip of the growing pollen tube, the response is triggered and growth is arrested. A role for IP_3-induced calcium release (IICR) has been demonstrated in this system. Similarly, in the case of cell volume control of *Fucus* rhizoids, a calcium wave-like characteristics could be observed. In response to hypoosmotic shock, the cells develop a tip-focused gradient that lasts for 1 to 2 min in the apical region only due to the influx of Ca^{2+}. This gradient moves like a slow wave to subapical regions using Ca^{2+} released from intracellular spaces (Taylor et al., 1996).

2. Calcium Oscillations

Oscillations are very specific signals that show that the regulation of cytosolic calcium is subject to strong feedback. From studies in animal systems, it is known that oscillations are repetitive pulses of high Ca^{2+} in response to some signal at a particular space only. Like waves, oscillations also have some functional significance because they act as filters to discriminate true signal from inherent environmental noise as shown in the animal system (Meyer and Stryer, 1991). In addition, they enhance the fidelity of signal perception by allowing signal integration over a long period of time yet without causing a deleterious sustained rise of cytosolic calcium (Tsien and Tsien, 1990). Oscillations could be symmetrical or asymmetrical, they could have a time period of few seconds to several minutes, and they are very much affected by extracellular Ca^{2+} levels.

In plants, oscillatory patterns of Ca^{2+} changes are best exemplified in stomatal guard cells in response to external signals where external Ca^{2+} causes an increase in $[Ca^{2+}]_{cyt}$ in an oscillatory manner (McAinsh et al., 1995). Oscillation patterns are different under different concentration of extracellular Ca^{2+}. When cells are incubated in 0.1 m*M* Ca^{2+}, symmetrical oscillations of a time period of 5 to 17 min, and an amplitude of 300 to 560 nm are observed, while incubating cells in the 1 mM $[Ca^{2+}]_{ext}$ oscillations were asymmetric with an amplitude of 400 to 850 nm and a time period of 10 to 16 min. This drastic difference in the pattern of oscillations based solely on the $[Ca^{2+}]_{ext}$ shows that the strength of the stimulus could give rise to a definite pattern of oscillations that in turn effects particular downstream pathways, giving specific responses. This shows that different sig-

nals would create different fingerprints for calcium thereby providing the specificity. Recently, it has been demonstrated that ABA can induce the calcium oscillations in the guard cell, and the pattern of oscillations is correlated with concentration of ABA used as well as the aperture of the guard cells. These ABA-induced oscillations are mediated by PI-PLC (phosphoinositide-phospholipase C) as the oscillations could be inhibited by U73122, a specific inhibitor of PI-PLC (Staxen et al., 1999).

Another very interesting pattern of Ca^{2+} change was observed in the case of nodulation. Ca^{2+} has been shown to be involved in the process of nodulation in alfalfa roots and on interaction with *nod* factors specific Ca^{2+} spikes are produced (Ehrhardt et al., 1996). These spikes show temporal as well as spatial regulation, that is, the oscillations that started at the narrow zone near the nucleus later propagated radially. All these processes show that achievement of specificity is possible by specific patterns of Ca^{2+} changes.

In addition to the spatial and temporal patterns of calcium changes providing specificity to the calcium signaling, the combination of different stresses can produce novel calcium signatures. Knight et al. (1998) very clearly demonstrated that by comparing mannitol stressed plants, pretreated with either osmotic or oxidative stress, the calcium responses are markedly altered and it depends on the identity and severity of previous stress. This again suggests that altered Ca^{2+} response encodes a memory of previous stress encounters and thus may be involved in acclimation to different stresses.

D. Calcium Binding Proteins — Mediators of Ca^{2+} Signaling

Changes in calcium levels are perceived by various calcium binding proteins that either by themselves or by modifying some other proteins and/or factors, transduce the calcium signal downstream to elicit the final response (Roberts and Harmon, 1992). Calcium ions have a coordination sphere of 6 to 8 oxygen atoms around them, which is involved in binding to various proteins. Based on the mechanism through which the proteins bind to Ca^{2+}, they can be divided into two classes, that is, proteins that use side chain carbonyl oxygen atom from the peptide backbone and contain an EF hand motif and proteins that use peptide backbone carbonyl oxygen for binding to Ca^{2+} (Moews and Kretsinger, 1975). On the functional basis, the Ca^{2+}-binding proteins could be engrouped as trigger proteins and buffer proteins. The trigger proteins are those that after binding to Ca^{2+} get activated and bring about the next step in signal transduction pathway. These proteins possibly get activated either by exposure of their unexposed domains due to change in conformation after Ca^{2+} binding or by the binding of Ca^{2+} to the inhibitory domain. Best examples of trigger proteins are calmodulin and Ca^{2+}-dependent kinases. Besides, a number of other plant CaBPs have been shown to respond to various signals (Frandsen et al., 1996). Buffer proteins bind to elevated calcium level just to sequestrate it and earlier suggested to have no other function. However, recent studies with calreticulin, one of the most common examples of buffer proteins, have shown that its level is developmentally regulated. Besides, under *in vitro* culture conditions it is also dependent on auxin levels. Localization studies have shown that the calreticulin has a tissue specific distribution pattern and at the subcellular level it is mostly localized in golgi bodies, indicating it has some important functional role in plants (Borisjuk et al., 1998). A large number of such proteins are found in plants (Krause et al., 1989; Menegazzi et al., 1993; Chen et al., 1994; Caughlan et al., 1997) and might have some yet unexplored roles to play in signaling processes, besides maintaining the calcium homeostasis of the cell (Nelson et al., 1997). Our attention in this review is mostly on trigger proteins.

E. Calmodulin

Calmodulin is the most well-characterized Ca^{2+} binding protein from plant system and is a prototype of EF hand family of proteins. It is a small heat-stable protein (MW 16.7 kDa) consisting of 148 amino acid residues. It contains four equivalent Ca^{2+} binding sites with k_d of 2.4 μM (Means et al., 1982). X-ray crystallography has

shown that it is a dumbbell-shaped structure (Babu et al., 1988). It contains two globular domains, each containing two Ca^{2+} binding sites connected by an α helix. Binding of Ca^{2+} to CaM is cooperative (Linse et al., 1991). CaM is an essential protein as knock-out mutations in this gene are lethal in *S. pombe* (Takeda and Yamamoto, 1997). Blocking of all four of its Ca^{2+} binding sites did not abolish the ability of protein to support growth showing that it has other functions as well besides binding to Ca^{2+} (Geiser et al., 1991).

CaM is a highly conserved, multifunctional protein and comparison of various cDNA sequences and alignment of amino acid sequences from various plant and animal CaM show a very high degree of homology, giving clues about the existence of a parallel Ca^{2+}/CaM signaling pathway in plants and animals.

The involvement of CaM has been shown during processes of cell cycle (Vantard et al., 1985), cell growth, and embryogenesis (Oh et al., 1992) germination of seed embryo (Cocucci and Negrini, 1988), differentiation of treachery elements (Kobayashi and Fukuda, 1994), cell proliferation (Perera and Zielinski, 1992), and phytochrome-mediated pathways (Lam et al., 1989; Neuhaus et al., 1993). Besides, responsiveness of CaM gene(s) to various stimuli and their spatially and temporally regulated expression also confirms its importance in Ca^{2+} signaling (Galaud et al., 1993).

Plants contain a large number of CaM isoforms in contrast to animal systems, where no isoform could be detected despite the presence of a multigene family. The presence of multigene family of CaM in plants, that is, two each in rice, *petunia,* and *vigna*; three in maize; five in soybean; six in *Arabidopsis;* eight in potato and the existence of a number of isoforms, that is, four in *Arabidopsis*; three in wheat and four in soybean (Ling et al., 1991; Liu et al., 1991; Gawienoiwski et al., 1993; Lee et al., 1995; Takezawa et al., 1995; Yang et al., 1996), possibly contributes to the diversity and specificity of Ca^{2+}/CaM-mediated signaling.

The expression pattern of CaM is spatially regulated as well. In the case of *Arabidopsis*, ACAM1 was most abundant transcript in leaves and developing siliques (Ling et al., 1991), while ACAM3 was expressed preferentially in aerial tissues except floral buds (Perera and Zielinski, 1992a; Antonsiewicz et al., 1995). The level of ACAM4, 5, and 6 were considerably lower than the other isoforms. In roots, only ACAM1 could be detected (Poovaiah and Reddy, 1991). In *Brassica* also, the level of CaM transcript was higher in leaves and shoot apical meristem than in root tips or root elongation zone (Chye et al., 1995). Maize CaM isoforms ZMCAM1 and ZMCAM2 were differentially expressed in different tissues (Breton et al., 1995). Similarly, in potato PCM1 isoform was highly expressed in stolon tip, moderately in roots, and very low in leaves, while PCM6 showed a steady-state expression level in all the tissues except roots (Takezawa et al., 1995). The expression of CaM mRNA was also found to be different in different tissues of soybean (Lee et al., 1995). In *Arabidopsis*, a study of transcriptional activation of six CaM genes in response to touch show that the two of the isoforms are not regulated by touch, whereas the other four show differences in the response (Verma and Upadhyaya, 1998). This shows that level of expression of CaM varies in different parts of a plant, and the isoforms also behave differently, thus providing the much needed diversity and specificity to Ca^{2+} signal.

Various stimuli, which act by modulation of Ca^{2+} concentration, have also been shown to effect the expression of different CaM-related gene(s). In the case of mechanical signals such as rain, wind, and touch, differential expression of various CaM-related genes was observed (e.g., TCH1–5 of *Arabidopsis*), and the pattern of expression of these genes was spatially and temporally regulated (Braam and Davis, 1990; Ito et al., 1995; Braam et al., 1997). Temperature stress also modulated TCH gene expression (Braam, 1992; Xu et al., 1995, 1996; Polisensky and Braam, 1996). Various other external stimuli as light (Jena et al., 1989; Braam and Davis, 1990; Botella and Arteca, 1994), auxin (Jena et al., 1989; Botella and Arteca, 1994; Okamoto, 1995), and ethylene (Braam and Davis, 1990), were also shown to bring about differential expression of CaM and CaM-related gene(s). Similarly, in the case of barley aleurone protoplast where GA and ABA effects are mediated by Ca^{2+}, a modulation of

CaM gene expression and protein level could be detected (Gilroy, 1996; Schuurinka et al., 1996). Similarly, Luit et al. (1999) have shown very clearly that calcium signals generated via wind and cold stimuli operate through CaM gene expression, and there is clear spatial separation of the pathways operated during both the stimuli. Recently, Heo et al. (1999) have shown that specific soybean CaM isoforms SCaM-4 and SCaM-5 are activated by infection or pathogen-derived elicitors and participate in Ca^{2+}-mediated induction of plant disease resistance responses, whereas other SCaM genes encoding highly conserved CaM isoforms did not show any effect.

F. CaM-Binding Proteins

Activation of a large number of other proteins besides kinases, such as nitric oxide synthase, myosin light chain kinase, phosphorylase kinase, calcineurine phosphatase, plasma membrane Ca^{2+} ATPases, and many other enzymes (Billingsley et al., 1990) are also regulated by CaM. Role of these proteins has been reviewed extensively recently (Zeilinski, 1998). In the following pages we have given only some specific examples where the proteins have been shown to be involved in calcium-mediated signaling.

It was shown that small nuclear NTPases get stimulated by CaM. As these nuclear NTPases also get stimulated during phytochrome signaling, it was postulated that CaM may be playing a role along with Ca^{2+} in phytochrome-mediated signaling through nuclear NTPases (Hsieh et al., 1996).

Another important protein with which CaM binds and stimulates its activity is glutamate decarboxylase (GAD). This enzyme has been purified from different plants (Ling et al., 1994) and shown to exist as many isoforms. GAD catalyzes decarboxylation of glutamate, thereby producing CO_2 and γ-amino butyrate (GABA), a very important component of many metabolic pathways. Transgenic petunia plants harboring a mutant GAD lacking the CaM binding site showed severe abnormalities confirming CaM regulation of GAD activity (Arazi et al., 1995). As GAD activity has been shown to get stimulated by hypoxia and some other stress signals, it indicates that besides its role in controlling various metabolic processes, it might also be involved in CaM-regulated stress signaling (Baum et al., 1993, 1996; Gallego et al., 1995; Snedden et al., 1996).

In earlier studies NAD kinases were found to be regulated by CaM in plants, whereas in animal systems no such regulation was observed. Recently, it has also been shown that NAD kinases are differentially regulated by CaM isoforms (Lee et al., 1995). In certain cases, light and some other stimuli have been shown to stimulate the activity of NAD kinases. Using transgenic plants that overexpress CaM and CaM mutants and by giving different stress or elicitor signals such as cellulase, heparin, and osmotic or mechanical stress to such transformed plants, it was found that CaM is the target of Ca^{2+} fluxes, and NAD kinase could be the downstream target for this Ca^{2+}/CaM-mediated signaling pathway (Harding et al., 1997; Lee et al., 1997).

The other important CaM binding proteins are the endoplasmic reticulum and tonoplast located Ca^{2+} ATPases and slow vacuolar ion channels that have been shown to be involved in a number of calcium signaling processes (Askerlund, 1997; Harper et al., 1998).

By using ^{35}S labeled CaM, it was found that it can bind to various microtubular motors in specific ways. A novel kinesin-like protein was isolated from *Arabidopsis* (Reddy et al., 1996a, 1996b, 1997) and tobacco (Wang et al., 1996). This kinesin-like CaM binding protein (KCBP) is distinct from all other KLPs in having a CaM binding domain adjacent to its motor domain and appears to be ubiquitous in plants. KCBP has been shown to interact with tubulin subunits (Song et al., 1997). The binding of KCBP with tubulin is regulated by Ca^{2+}/CaM, that is, in the presence of Ca^{2+}/CaM the motor with CaM binding domain does not bind to tubulin, and this modulation is abolished in the presence of antibodies specific to CaM binding domain of KCBP. This CaM-dependent modulation of KCBP interaction with tubulin suggests the regulation of the KCBP function by calcium (Narasimhulu et al., 1997). KCBP has also been shown to have a role in trichome morphogenesis (Oppenheimer et al., 1997) as well as in cell division. During cell division, these

proteins have been found to be associated with preprophase band, mitotic spindle, and phragmoplast. Association with these microtubular motor arrays in dividing cells suggests that this negative end-directed motor protein is likely to be involved in the formation of microtubular arrays and/or functions associated with these structures (Bowser and Reddy, 1997). The myosin heavy chain binding cDNA also contains a putative CaM binding site (Kinkema and Schiefelbein, 1994). This shows that CaM is involved in intracellular transport processes in cells. The role of CaM has also been shown in gravitropic responses in *Arabidopsis thaliana* agr-3 mutants (Sinclair et al., 1996). Recently, glyoxalse enzyme, which has been reported to be involved in cell proliferation (Deswal et al., 1993) and in stress responses (Veena et al., 1999), has been shown to be a calcium/calmodulin-binding protein (Deswal and Sopory, 1999).

Some other proteins, such as heat shock proteins, have also been shown to contain CaM binding domains (Li et al., 1994; Lu et al., 1995). In addition, few transcription factors of basic helix-loop-helix family (Corneliussen et al., 1994) and basic amphipathic α-helix (Dash et al., 1997) also contain CaM binding domains. In *Arabidopsis* ACAM3 promoter binds to the leucine zipper family of transcription factor TGA3, while CaM itself acts as an enhancer of TGA3 binding with its *cis* elements (Szymansky et al., 1996). Different CaM isoforms differentially enhance binding of TGA3 promoter to ACAM3 and also to cauliflower nuclear proteins. All this suggests that Ca^{2+}-mediated signaling coupled to gene expression might go via CaM and CaM binding transcription factors and could lead to specificity of the response.

G. Protein Kinases

The role of kinases and phosphatases in plant signaling processes has been reviewed recently (Sopory and Munshi, 1997). In this review we are focussing on the calcium-mediated processes where involvement of these proteins is known, along with a brief discussion on their characteristic properties.

Kinases are the main trigger proteins of the Ca^{2+} signaling pathway. Changes in the level of Ca^{2+} in response to various stimuli alter the phosphorylation pattern of various proteins that are the main mediators in the signal-response coupling.

A large number of protein kinases have been shown to exist in plants, which are classified into various groups, subgroups, families, and subfamilies (Stone and Walker, 1995). Some of these kinases are similar to the kinases present in animal systems, while others are specifically present in plants. Calcium regulates three different families of protein kinases in plants, viz., calcium-dependent protein kinases, which require only calcium for activity, Ca^{2+}/CaM-dependent kinases, which along with Ca^{2+} also require CaM for their activity, and Ca^{2+}/lipid-dependent kinases, which require lipids along with Ca^{2+} for activity.

1. Calcium-Dependent Protein Kinases (CDPKs)

These are widely distributed and well-characterized kinases, and their existence has been shown in many plants, some protists (Gunderson and Nelson, 1987; Farber et al., 1997), and green algae (Guo and Roux, 1990; Yuasa and Muto, 1992; Yuasa et al., 1995). CDPKs are plant-specific protein kinases, as no CDPK homologue has been reported from the animal systems as yet. These kinases require micromolar concentration 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 C terminal autoregulatory domain and a CaM-like domain (CaMLD), which has Ca^{2+} binding EF hand or helix-loop-helix motif (Battey and Venis, 1988; Harper et al., 1991; Suen and Choi, 1991). The autoinhibitory domain of CDPKs is a 30 amino acid sequence, which is a pseudo-substrate inhibitor (Harper et al., 1994). The N-terminal domain of CDPKs is variable, and it provides specificity to different CDPK isoforms. These enzymes show severalfold stimulation with Ca^{2+} and show autophosphorylation. They are both soluble as well as membrane bound and have been reported from organelles also. Although CaM does not stimulate these kinases, different CaM inhibitors affect the activity of these CDPKs, possibly due to the existence of CaMLD. The

autoregulatory domain keeps the activity of these kinases at the basal level and a mutation in this domain produces constitutively active enzyme (Huang et al., 1996). Under *in vitro* conditions the kinases gets activated by binding to Ca^{2+} and use various proteins as substrates like histone, casein, phosvitin, BSA, and few synthetic peptides, but *in vivo* substrates are not known in many cases. Genes for various CDPKs have been cloned, and some of them are coded by a multigene family (Biermann et al., 1990; Ali et al., 1994; Breviario et al., 1995; Thummler et al., 1995; Hrabak et al., 1996; see Redhead and Palme, 1996).

2. Ca^{2+}/CaM-Dependent Protein Kinases

These are a group of calcium-dependent kinases, which in addition to calcium also require CaM for their activity. Thus CaM, besides acting directly, could also exert its effects by binding to protein kinases and modulating their activities. In animal systems, CaM has been shown to activate Ca^{2+}/CaM-dependent kinase I, II, and III, that regulate a wide variety of physiological processes involving Ca^{2+}-mediated signaling (Colbran et al., 1989). As the importance of Ca^{2+}/CaM kinases in animal systems is very well established, attempts were made to look for a parallel signaling pathway in plant systems as well. Some indirect studies (Salimath and Marme, 1983; Blowers et al., 1985; Blowers and Trewavas, 1985, 1989) predicted the existence of this family of kinases in plants but it was confirmed only after the cloning of various cDNA homologues from different plant systems, for example, carrot (Suen and Choi, 1991), apple (Watillon et al., 1992, 1993, 1995), lily (Patil et al., 1995; Takezawa et al., 1996), and maize (Lu et al., 1996, 1997). All these homologues show a considerable similarity with their animal system counterparts at the cDNA level.

The kinase cDNA homologue from apple encodes a single polypeptide (MW 46.5 kDa) with serine/threonine catalytic domain and an adjacent Ca^{2+}/CaM-binding regulatory domain. It shows considerable homology to the corresponding regions of mammalian multifunctional Ca^{2+}/CaM protein kinases II (Watillon et al., 1995).

Ca^{2+}/CaM-dependent kinase gene from lily anthers is a chimeric gene containing a neural visinin-like Ca^{2+}-binding domain fused with a CaM-binding domain. The amino terminal region of the encoded protein contains all 11 conserved subdomains characteristic of serine/threonine protein kinase. The CaM binding region has high homology (79%) to a subunit of mammalian Ca^{2+}/CaM-dependent protein kinase (Patil et al., 1995). Biochemical properties of lily Ca^{2+}/CaM-dependent kinase have been studied by over expressing its cDNA in *E. coli*. The biochemical properties show that it is a nonconventional, novel Ca^{2+}/CaM kinase. The kinase shows a dual regulation with Ca^{2+} and CaM, as autophosphorylation of the protein is only Ca^{2+} dependent, while substrate phosphorylation is regulated by both Ca^{2+} and CaM, although neither of them modulate the kinase activity individually (Takezawa et al., 1996). Using the yeast two hybrid system to obtain genes coding for the proteins interacting with this kinase, a cDNA clone has been obtained that shows very high similarity to elongation factor-1 alpha (EF–1 alpha). The kinase phosphorylates EF–1 alpha in a calcium/CaM-dependent manner, suggesting its direct role in the regulation of gene expression (Wang and Poovaiah, 1999). Similar CCaMK genes have also been cloned from tobacco (TCCaMK–1 and TCCaMK–2), and these genes show differential regulation by calmodulin isoforms (Liu et al., 1998).

MCKI, a Ca^{2+}/CaM kinase homologue isolated from maize roots (Lu et al., 1996, 1997) contains all 11 conserved subdomains characteristic of protein kinase catalytic domain and all the conserved amino acid residues. It shares sequence homology with yeast CMK1 (42%), rat CaMKII (37%), and apple CBI (34%). This kinase is expressed in root caps, a site of perception of both light and gravity signals. Because MCKI is expressed both in light and dark grown tissue, it appears not to be directly regulated by light, but has been shown to be involved in gravitropic responses. However, biochemical properties of this kinase have not been studied.

We have purified a Ca^{2+}/CaM-dependent kinase from *Zea mays* coleoptiles. This kinase has a molecular weight of 72 kDa and has been characterized biochemically. This kinase belongs to the serine/threonine family of protein kinases and shows a dual regulation by calcium and CaM. The substrate phosphorylation is calcium dependent,

and addition of exogenous CaM stimulates it further, whereas autophosphorylation is only calcium dependent (Pandey and Sopory, 1998). The kinase is involved in light- and stress-mediated signaling (Pandey and Sopory, unpublished).

All these studies regarding cDNA homologues of Ca^{2+}/CaM-dependent kinases show the presence of both conventional and novel Ca^{2+}/CaM-dependent protein kinases in plants systems. As these kinases are involved in a wide range of signaling processes in animal systems, their important role is also envisaged in Ca^{2+} signaling pathways in plants.

3. Ca^{2+}/Phospholipid-Dependent (PKC-T6ype) Protein Kinases

PKC-type kinases require phospholipids for their activity, in addition to Ca^{2+}. These kinases belong to the serine/threonine family of protein kinases. In mammalian system they are basically involved in various regulatory processes (Nishizuka, 1992), playing a pivotal role in signal transduction involving receptor-mediated hydrolysis of $PIP_{2,}$ which produces IP_3 and DAG. DAG is the activator of PKC-type kinases, while IP_3 releases calcium from intracellular stores. The presence of these kinases has been shown from both membrane and soluble fraction, and they show some selectivity toward the lipid they require for stimulation. In plants, some of the purified kinases have been shown to have similar activities as their animal system counterparts (Baron-Marting and Scherer, 1989; Komatsu and Hirano, 1993; Honda et al., 1994; Nanmori et al., 1994; Karibe et al., 1995; Chandok and Sopory, 1998). Although these kinases have been reported from various plant systems, their exact role in calcium-mediated signaling is not known, and further work is needed in this direction. We have characterized in great detail cPKC activity in maize and shown its role in light-mediated nitrate reductase (NR) gene induction (Chandok and Sopory, 1998).

4. Kinases and Calcium-Dependent Signaling

Ca^{2+}-dependent kinases are the most widely studied and characterized components that couple changes in Ca^{2+} level to a specific response and have been described earlier. Most of the Ca^{2+} signal transduction processes are mediated by a change in the phosphorylation/dephosphorylation pattern of related proteins (Huber et al., 1994; Monroy et al., 1997). In some cases, the concerned Ca^{2+}-dependent kinases are identified and characterized. Signals such as light (Otto and Schaffer, 1988; Romero et al., 1991; Reymond et al., 1992; Fallon et al., 1993; Harter et al., 1994a,b; Short and Briggs, 1994; Sharma et al., 1997a,b), auxin (Poovaiah et al., 1988; Botella et al.,1996; Yang and Poovaiah, in press), GA (Abo-El Saad and Wu, 1995; Hutty and Philips, 1995; Chen et al., 1997; Ritchie and Gilroy, 1998), cytokinins (Hepler and Wayne, 1985; Sano and Youssefien, 1994), abscisic acid (Holappa and Walker-Simmons, 1995; Hwang and Goodman, 1995; Hey et al., 1997), ethylene (Fluhr and Mattoo, 1996; Kim et al., 1997), various stresses (Anderberg and Walker-Simmons, 1995; Holappa and Walker-Simmons, 1995; Hwang and Goodman, 1995; Bogre et al., 1996; Botella et al., 1996; Pasenacz and Erdei, 1996; Sheen, 1996), elicitors (Felix et al., 1991, 1994; Suzuki and Shinshi, 1995; Xing et al., 1996), pathogens (Martin et al., 1993; Vera-Estrella et al., 1994; Loh and Martin, 1995; Song et al., 1995; Zhou et al., 1995), sugar (Ohto and Nakamura, 1995), and nitrate (Bachmann et al., 1995) have been shown to mediate their responses via a change in phosphorylation pattern of proteins. In few cases the evidence for involvement of CDPKs in signaling exist. Most of these studies are based on the facts that transcript level of the CDPK homologues change in response to a particular stimulus. In the case of wheat embryo, a strong up-regulation of a transcript PKABA1 was observed in response to ABA and dehydration (Anderberg and Walker-Simmons, 1992). The transcript accumulated under dehydrated conditions, indicating that it might be the initial response of plant to stress. Similarly, in mung bean expression of VrCDPK1 is up-regulated by auxin (Botella et al., 1996). The level of WPK4mRNA, a kinase homologue from wheat, is up-regulated in response to cytokinin (Sano and Youssefien, 1994). Various stress signals that act through the modulation of $[Ca^{2+}]_{cyt}$ level, act by modulating CDPKs (Sheen, 1996). Using chimeric constructs

containing ABA-responsive promoter element and GFP reporter protein, it was observed that signals such as cold, salt stress and ABA cause an expression of reporter gene when protoplasts are incubated in Ca^{2+}. Co-expression of CDPKs resulted in an increase in reporter gene expression that was abolished by using mutated CDPKs or phosphatases, showing conclusively that CDPKs are involved in stress regulated signaling (Sheen, 1996). Some other processes which involve a change in $[Ca^{2+}]_{cyt}$ level as nodulation, a nodule specific CDPK has been reported which might be involved in the signaling process. Activity of specific CDPKs has also been found to change during the process of *in vitro* tuberization of potato (MacIntosh et al., 1996) and hypo-osmotic turgor regulation in Characeae (Takashi et al., 1997), both of which are preceded by a $[Ca^{2+}]_{cyt}$ level change. A pollen-specific CDPK has been cloned and characterized and its transcription is regulated to the stage of pollen development (Estruch et al., 1994).

H. Protein Phosphatases

Dephosphorylation reactions catalyzed by protein phosphatases are as important as phosphorylation reaction in Ca^{2+} signaling (Shenoliker, 1994; Hunter, 1995). The role of protein phosphatases is not as well studied as that of protein kinases in Ca^{2+}-mediated signaling in plants; nevertheless, their importance cannot be underestimated.

Protein phosphatases have been classified into two classes (Cohen et al., 1990):

Protein phosphtase-1 (PP1), which dephosphorylate the β subunit of the mammalian phosphorylase kinase and inhibited by endogenous protein inhibitors 1 and 2.

Protein phosphatase-2 (PP2), which dephosphorylate the α subunit of the phosphorylase kinase and are resistance to inhibitors 1 and 2 (Smith and Walker, 1996). PP2 could be subdivided into three groups PP2A, PP2B, and PP2C. PP2A is a heterotrimer containing a catalytic C unit and a regulatory A and B subunit and does not require divalent cations for its activity. PP2B is a heterodimer, containing a catalytic C subunit and a regulatory A subunit. It requires Ca^{2+} for its activity, and it belongs to the EF hand family of proteins. PP2C is a monomeric protein and requires Mg^{2+} for its activity.

In mammalian systems the catalytic subunit of different phosphatases are products of distinct genes and, although PP1, 2A, and 2B are related, PP2C has no structural homology with them. Most of the plants show a presence of a multigene family for protein phosphatases and show differential expression in different plant parts; however, their role in calcium signaling remains to be elucidated (MacIntosh and Cohen, 1989; Jogiello et al., 1992; Arino et al., 1993; Casamayor et al., 1994; Evans et al., 1994; Li et al., 1994; Pay et al., 1994; Rundle et al., 1995; Corum et al., 1996; Guo and Roux, 1996; Hammer et al., 1997; Latorre et al., 1997).

1. Phosphatases and Calcium-Dependent Signaling

Plant phosphatases have been shown to be involved in many signal transduction pathways (Arundhati et al., 1995; Zimmerlin et al., 1995), but very few studies have been reported in processes mediated via Ca^{2+}. One example of involvement of protein phosphatase in signaling in plant cells is in stomatal guard cells, where calcineurin, a type 2B phosphatase, modulates the activity of slow vacuolar ion channels (Allen and Sanders, 1995). In addition, a cDNA clone isolated from plants could complement for calcineurin mutation in yeast and increase salt tolerance in wild-type yeast (Lippuner et al., 1996).

One of the most studied signaling pathway mediated through phosphatase is ABA signaling. ABA has been shown to control many aspects of plant growth and development, and the ABI gene (*ab*scisic acid *i*nsensitive) has been shown to mediate many ABA-regulated responses. This gene codes for a Ca^{2+}-modulated protein phosphatase (Leung et al., 1994; Meyer et al., 1994). Complementation studies in yeast and biochemical characterization of the protein has further confirmed this ABI1 protein to be a PP2C homologue (Bertauche et al., 1996). Leung et al. (1996,

1997a,b) have isolated another ABI homologue, ABI–2, which also code for a PP2C homologue. Both these homologues maintain a partially redundant function in ABA signaling. Ca^{2+}-mediated protein kinase-phosphatase pathway has been shown to be involved in rice aldolase C1 gene expression (Nakamura et al., 1996).

Oligogalactoronidase signaling in plants is also regulated by phosphatases in *Nicotiana tabaccum*. Oligogalactoronidase modifies the protein phosphorylation pattern of several polypeptides. One of these proteins is calreticulin, the main Ca^{2+}-sequestering protein of ER, and its phosphorylation is increased in the presence of okadaic acid, a phosphatase inhibitor. This shows that calreticulin could be functional in an early signal transduction pathway involving a protein phosphatase (Droillard et al., 1997).

Involvement of phosphatases has also been shown in TMV-mediated programmed cell death and PP1 is required for early events of host response to virus infection (Dunigan and Madlener, 1995). Phosphatases are also involved in light-mediated expression of several genes (Sheen et al., 1993), besides several chloroplast proteins also require rapid dephosphorylation in various light-mediated processes (Elich and Chory, 1997; Hammer et al., 1997). A role for PP2A has been suggested in auxin transport in *Arabidopsis* (Garbers et al., 1996), and a novel protein phosphatase KAPP (kinase associated protein phosphatase) has been shown to be involved in signaling process mediated by RLK (receptor-like kinase). Involvement of an extraplastidic serine/threonine protein phosphatase and protein kinase has also been shown in plastid transcription (Cristopher et al., 1997), and tyrosine dephosphorylation of histidine kinase in cytokinin control of cell cycle (Zhang et al., 1996). In addition, a novel plant oncogene has also turned out to be a plant phosphatase (Filippini et al., 1996), but a role for calcium in these processes is not clear.

CONCLUSIONS

The presence of an efficient calcium homeostasis system and the involvement of calcium in many physiological responses have conclusively proven calcium as the most important signaling molecule. Calcium ions regulate these responses in a very specific manner, and this specificity is achieved at different levels. The source of calcium and type of cell responding to a particular signal confers the specificity at the primary level. At other levels, specificity is achieved by the strength of the calcium signal and the specific patterns of calcium change, that is, varying from a small transient increase to complex patterns of ramping, oscillations, spikes, and waves. Another level of control is achieved by changes in activity of various other proteins and factors that get activated or modulated by changes in calcium levels. Calcium-dependent kinases and phosphatases belong to this category and are main components of signal response coupling. Besides, changes in level of other factors in concert with calcium (e.g., CaM, DAG, etc.) also activate a specific set of proteins, and this could also be involved in contributing the specificity of signaling. Calcium directly affecting expression of certain genes gives another level of control on specificity. Other components are specific channels, transporters, and some motor proteins that also regulate calcium signaling. Recently, a mutant has also been identified showing altered calcium response that couldd also help deciphering the mechanism of calcium action in plants (Liu and Zhu, 1997).

Thus, all these facts indicate that cellular calcium signaling is a very complex yet precise phenomenon and involves interaction of a large number of components. Although some progress has been made to understand the wide variety of processes it regulates with a precise specificity, more work is required in this direction to pinpoint the exact role and mode of action of calcium in particular processes.

NOTE ADDED IN PROOF

A new family of calcium sensors in *Arabidopsis* that are similar to both the regulatory B subunit of calcineurin and neuronal calcium sensor in animals have been characterized and shown to be affected by stress signals (Liu and Zhu, 1998; Kudlas et al., 1999). Like proteins, *Arabidopsis caldineurin* may be transducing the singals through a group of novel protein kinases reported recently (Shi et al., 1999).

Another novel kinase that does not belong to the known categories was purified from *Brassica juncea* and found to be stimulated by calcium and calcium-binding protein from *Entamoeba histolytica* whose homologs were detected in plants (Deswal et al., 2000).

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