Signal perception and transduction are important events in plants, as plants cannot escape from the environment, they live in like animals. All organisms perceive the stimuli and trigger the signal transduction pathways. Therefore, understanding the molecular biological events are important in stress responses of plants. A brief review on calcium as a signalling molecule is presented here.

**Calcium is a versatile messenger in signalling**

All organisms use a network of signal transduction pathways to cope with their environment, to control their metabolism and to realize their developmental programs. Calcium has emerged as an ubiquitous second messenger involved in many of these processes. Plant cells are equipped with highly efficient mechanisms to perceive transducer and respond to a wide variety of internal and external signals during their growth and development. Perception of signals via receptors results in generation or synthesis of non-proteineous molecules often termed messengers. These messengers control diverse cellular processes through sensors (proteins/enzymes) (Bower and Fluhr, 2000; Reddy, 2001; Rudd and Franklin-Tong, 2001; Sander et al., 2002; Sendden and Fromm, 2001; White and Broadley, 2003; Yang and Poovaiah, 2003). The elements of receptors, messengers, sensors and targets vary depending on the signal. Identification and functional assignments of these elements in a stimulus-specific signal transduction pathway is a challenging area for plants biologists (Bowler and Fluhr, 2000; Romeis et al., 2001; Zhu, 2003)
Environmental and hormonal stimuli affect many aspects of growth and development processes in plants. Many of these signals evoke specific responses in plants. Because plant cells respond to great variety of stimuli, there must be built in mechanisms to sense and transduce the signals. In recent years understanding signal transduction pathways - the series of biochemical and molecular events that are involved in evoking a final response, have become one of the foremost areas of plant biology research. In the late 1970s, following the discovery of calmodulin (CaM), a Ca\(^{2+}\) -binding protein, and CaM dependent enzymes, it was proposed that Ca\(^{2+}\) may act as messenger in signal transduction in plants. The realization that Ca\(^{2+}\) may play messenger role has resulted in increased interest in Ca\(^{2+}\) research (Hepler, 1997; Poovaiah and Reddy 1993; Trewavas and Knight, 1994).

Ca\(^{2+}\) should meet the following four criteria in order to consider it to be a messenger in regulating the physiological processes evoked by primary stimuli (Hepler, 1997; Poovaiah and Reddy 1993): (1) cytosolic Ca\(^{2+}\) must change in response to primary stimuli and such a change should precede the physiological responses; (2) artificial induction of changes in cytosolic Ca\(^{2+}\) should evoke a physiological response in the absence of primary stimuli (3) cells must possess the mechanism to sense the changes in cytosolic Ca\(^{2+}\) and translate them into physiological response and (4) blocking changes in cytosolic Ca\(^{2+}\) or Ca\(^{2+}\)-sensing system must prevent the physiological response to external stimuli.

The concept that Ca\(^{2+}\) acts as a messenger is based on the notion that environmental and hormonal signals induce changes in cytosolic Ca\(^{2+}\). Hence, determining the changes in the level of cytosolic Ca\(^{2+}\) in response to external stimuli has been crucial in establishing the role of Ca\(^{2+}\) as a messenger in plants. Although there was circumstantial evidence that signals affect cytosolic Ca\(^{2+}\), it was not established until recently by direct Ca\(^{2+}\) measurement in the cytosol.
The efforts to measure cytosolic Ca\(^{2+}\) in plant cells using the methods that are routinely used in animal systems have met with limited success. This is mainly due to unique structural features of plant cells such as the cell wall and the large vacuole of plants and other problems such as dye compartmentalization, leakage, and toxicity (Bush and Jones, 1990; Poovaiah and Reddy, 1993). Use of Ca\(^{2+}\) - binding fluorescent dyes (quin-2, fura-2 and indol-1), Ca\(^{2+}\) -selective electrodes, and microinjection of aequorin have been successfully used. These methods have helped to establish that cytosolic Ca\(^{2+}\) in plant cells is maintained in the range of 0.1 to 1 \(\mu\)M and to demonstrate in some cases signal induced changes in cytosolic Ca\(^{2+}\) (Bush and Jones 1989,1990; Gilroy et al., 1987; McAinsh et al., 1998; Poovaiah and Reddy 1993; ). Some novel approaches, such as using transgenic plants that express apoaequorin (Knight et al., 1991) confocal microscopy and fluorescence imaging and acid - loading of fluorescence dyes (Bush and Jones 1989) have been developed that are successfully used in monitoring the changes in cytosolic Ca\(^{2+}\) levels in response to various external stimuli.

Recent studies revealed that various non-proteineous molecules serve as messenger in conveying signals to cellular machinery in plants (Reddy, 2001). The messengers include Ca\(^{2+}\) ions, small organic molecules such as cyclic nucleotide monophosphate, inositol tri- phosphates and inorganic molecules such as hydrogen peroxides and nitric oxide (Demidchik et al., 2002b; Guo et al., 2003; Levine et al., 1994; Sanders et al., 2002; Scrase-Fied and Knight, 2003; Talke et al., 2003; Trewavas and Malho, 1997)

**The origin of the calcium signal**

There is a substantial amount of calcium in all eukaryotes cells but the resting concentration of calcium in the cytosol or free cytoplasm, \([Ca^{2+}]_c\), is extremely low, that is in the order of 0.1 \(\mu\)M or less. Most of the cellular calcium is bound either to proteins or in phosphate complexes in the mitochondria. The
precise concentration of free ions depends on the cell type and the measurement technique. The low intracellular calcium concentration is opposed to the calcium level in the extracellular fluids of mammals in which the concentration of free or weakly bound ions in most cases is 10,000-fold higher (Rasmussen, 1970). It has been proposed that the low intracellular level of calcium was selected through evolution because the phosphate-driven metabolism otherwise would involve the risk of precipitation of hydroxyapatite (\( \text{Ca}_5(\text{PO}_4)_3 \text{OH} \)) or other calcium phosphates of low solubility (Kretsinger, 1977). The differences in calcium concentration in the intra- and extracellular media creates a tremendous chemical gradient across the plasma membrane that far exceeds the gradients of Na\(^+\), K\(^+\), and Mg\(^{2+}\) (Martin, 1984). On top of this, because the interior of the membrane is negatively charged, there is an additional electrical gradient that favours entry of Ca\(^{2+}\) into the cells. To withstand the osmotic pressure introduced by the calcium gradient, the plasma membrane contains potent pumps that are capable of expelling calcium ions from the cytoplasm at the cost of energy. Among these, the calcium ATPase transports calcium to the outside at the cost of adenosine triphosphate (ATP), while calcium ions also can be expelled through the Na-Ca exchange system by letting in Na\(^+\) ions and that in turn would be transported out via the Na pump, again at the cost of ATP. Calcium is also equilibrated between the cytosol and various intracellular compartments such as the mitochondria and the endoplasmic or sarcoplasmic reticulum.

**Ca\(^{2+}\) Homostasis in the plant cytoplasm**

Cytosolic Ca\(^{2+}\) concentration in plant cells at rest is approximately 100-200 nM, which is typical of those found in all eukaryotes. The cytoplasm is bound on the outside by the Plasma membrane (PM), which is tightly appressed to the cell wall. These walls, especially in young tissue, are porous, allowing the diffusion of water, nutrients and molecules as large as 40 kDa (Carpita, 1982). Within the matrix of the wall, and often chelated by it, Ca\(^{2+}\) concentrations can be
high, \((10^{-4} \text{ to } 10^{-5} \text{ M})\) \cite{Cleland1990, Evans1991}. The cytoplasm is bound on the inside by the endomembrane system, which delineates numerous organelles. Of these, at least the vacuole and ER have \(\text{Ca}^{2+}\) concentrations higher than that of the cytoplasm. The vacuole may be particularly important as a repository for cell \(\text{Ca}^{2+}\), as it often accounts for 90-95\% of the cell’s volume and contains millimolar concentrations of \(\text{Ca}^{2+}\) \cite{Felle1988}. Luminal ER \(\text{Ca}^{2+}\) has rarely been measured in plants, but has been found to be above 3 mM in one case \cite{Bush1989}. There is evidence that proteinaceous \(\text{Ca}^{2+}\) chelators analogous to BiP are also present within the ER \cite{Jones1991}. Because the cytoplasm is surrounded by extensive regions of high \(\text{Ca}^{2+}\) and cytoplasmic \(\text{Ca}^{2+}\) must be maintained at nanomolar levels to support ATP-based metabolism, a variety of \(\text{Ca}^{2+}\) transporters are employed by plant cells to regulate cytoplasmic \(\text{Ca}^{2+}\).

**Ca\(^{2+}\)-ATPases**

\(\text{Ca}^{2+}\)-ATPases have been found in the PM (Plasma membrane), tonoplast and ER of plant cells. The functions of the PM and vacuolar \(\text{Ca}^{2+}\)-ATPases seem to be long-term maintenance of steady state \(\text{Ca}^{2+}\) levels \cite{Evans1991} and resetting of cytoplasmic \(\text{Ca}^{2+}\) concentrations \cite{Felle1992} by transport of \(\text{Ca}^{2+}\) from the cytoplasm to the apoplast or vacuole. The ER \(\text{Ca}^{2+}\)-ATPase may also serve this function \cite{Hepler1985} and also provide regions of high \(\text{Ca}^{2+}\) within the ER, which may be necessary for protein folding \cite{Sambrook1990}. Calcium co-transporters and antiporters have not been widely studied in plants, although H\(^+\)/ \(\text{Ca}^{2+}\) antiporters have been reported in the vacuolar membranes of oats \cite{Schumaker1990} and red beet \cite{Blackford1990}, presumably functioning in conjunction with the H\(^+\)-ATPases and H\(^+\)-PPIases that acidify the vacuole.
Ca\(^{2+}\) Channels

Several plant Ca\(^{2+}\) channels have been identified by their electro physical characteristics, but identification of Ca\(^{2+}\)-channel proteins has proved problematical (Harvey et al., 1989; Hetherington et al., 1992; Thuleau et al., 1993). They have been reported to open in response to voltage (Johannes et al., 1992b), stretch (Cosgrove and Hedrich, 1991), IP3 (Alexandre and Lasalles, 1990, 1992; Alexandre et al., 1990) and abscisic acid (ABA) (Schroeder and Hagiwara, 1990). As opposed to the Ca\(^{2+}\)- ATPases, Ca\(^{2+}\)channels in the PM, and perhaps in the tonoplast, seem to function in short-term modulation of cytoplasmic Ca\(^{2+}\) levels, often in response to extracellular factors. An IP3-regulated channel in the tonoplast may play a major role in the release of Ca\(^{2+}\) into the cytoplasm from intracellular stores, although this is yet to be demonstrated (Schroeder and Thuleau, 1991). The function of stretch activated Ca\(^{2+}\) channels is still to be established, but these may prove to be an integral part of the plants response to water availability, externally applied physical forces and growth.

Calcium binding proteins

The intermediary compounds between the modification of intracellular Ca\(^{2+}\) concentration in response to a stimulus and the physiological response are some type of Calcium-Binding Protein (CaBP). They are calcium dependent enzymes, channels, or calcium dependent modulating proteins. Other CaBP serve as Ca\(^{2+}\) buffers and do not participate in signal transduction. Therefore, CaBP are functionally divided in two categories:
**Calcium Sensors**

Calcium dependent enzymes (e.g. kinases, phosphatases, lipases, proteases), channels (e.g. \( \text{Ca}^{2+} \) gated channels, IP3R, RyR), modulators (activators, inhibitors) of the activity of target proteins (like calmodulin, troponin-C). Calcium sensors are proteins that transduce the \( \text{Ca}^{2+} \) signal to other cellular components. \( \text{Ca}^{2+} \) binding sensors is generally characterized by a fast kinetics and a relatively low affinity. Moreover with modulators, \( \text{Ca}^{2+} \) induces a conformational change resulting in the exposure of hydrophobic surface(s), allowing thereby interactions with other target proteins. \( \text{Ca}^{2+} \) sensors are characterized by an affinity for \( \text{Ca}^{2+} \) (KDa) in the range of \( 0.001 - 1 \, \text{mM} \) to bind physiological \( \text{Ca}^{2+} \).

**Calcium Buffers**

\( \text{Ca}^{2+} \) buffering proteins are in the cytosol and in the intracellular \( \text{Ca}^{2+} \) stores (e.g. parvalbumin, calsequestrin). They help to maintain “stabilized” \( \text{Ca}^{2+} \) concentration, and so play a role in the \( \text{Ca}^{2+} \) homeostasis. Binding of \( \text{Ca}^{2+} \)to buffer proteins is generally characterized by a slow kinetics and a relatively high affinity. Upon calcium binding they generally show no exposure of hydrophobic surfaces, and do not interact with target proteins. The buffer proteins play however an important role stabilizing \( \text{Ca}^{2+} \) concentration, allowing proper \( \text{Ca}^{2+} \) signal generation, and protecting the cell from \( \text{Ca}^{2+} \) overload.

To bind \( \text{Ca}^{2+} \), proteins possess different \( \text{Ca}^{2+} \) binding site structures. The same \( \text{Ca}^{2+} \) binding motif can often be found, as well in sensor as in buffer proteins so there is no strict correlation between site structure and sensor/buffer partition. The number of CaBP whose functions are known, such as calmodulin, troponin-C (Zot and Potter 1989), calpain (Murachi, 1989), Protein Kinase-C
(Asaoka et al., 1992), are far outnumbered by those whose roles are unknown, or elusive.

**Different types of CaBP**

On the basis of their cellular localizations and of the primary structure of their Ca\(^{2+}\) binding CaBP have been classified into different types:

**In extracellular CaBP**, Ca\(^{2+}\) act as stabilizing ligand for proper folding and constituents present in the protein. eg: some proteases, phospholipases, amylases, clotting factors, and growth hormones.

Intracellular CaBP, can be divided in 2 sub-types: Cytosolic CaBP, Intraorganellic CaBP.

**Cytosolic CaBP**, which can be divided in several groups depending on their calcium binding site structure:

**CaBP with EF-hand Ca\(^{2+}\) binding motif** (30 residues)

This is a very large family, with a high affinity for Ca\(^{2+}\) and a high evolutionary diversity (Persechini et al., 1989; Kretsinger and Nakayama, 1993). They play versatile roles in Ca\(^{2+}\) mediated cellular events and are mainly located within the cytoplasm and the nucleus although a few have been demonstrated to interact with membrane lipids when myristoylated. eg: calmodulin, troponin-C, parvalbumin etc.
**Ca^{2+}/ phospholipid-binding proteins**

Ca^{2+}/ phospholipid-binding proteins are also called annexin proteins (Burgoyne and Geisow, 1989). They have been suggested to mediate membrane fusion and to be involved in the control of cell proliferation and differentiation. Generally, they are localized in the cytoplasm and become translocated to the plasma membrane in response to an increase in the cytosolic Ca^{2+} concentration.

**CaBP with the C2 key Ca^{2+}/ phospholipids binding motif** (120 residues).

The C2 region (Shao et al., 1996) was found originally in (some) Protein Kinase C (PKC) isoforms. The physiological functions of C2 proteins are relatively well elucidated (Asaoka et al., 1992; Clark et al., 1991; Perin et al., 1990) as compared with EF-hand or annexin proteins. They often show Ca^{2+} induced translocation from the cytosol to membrane (like the annexins), and interaction with common cellular receptors. Example: Protein Kinase C, phospholipase C, etc.

**CaBP with other motifs**

Some Ca^{2+}/ actin binding proteins that serve the actin microfilament show common structural motifs. We can also consider Ca^{2+} gated channels as CaBP, but their Ca2+ binding domains are not defined and there is a possibility that their sensibility to Ca^{2+} is mediated by intermediary proteins. Example: Ca2+-gated K+ channels, IP3R, etc.
Inter-organellic CaBP, in ER and mitochondria.

The organelles buffer the concentration of Ca\(^{2+}\). They have no defined Ca\(^{2+}\) binding structural pattern and in general a low affinity and high capacity for Ca\(^{2+}\) binding (few EF-hand CaBP have also been identified in the ER). So far, there is only one report of a protein containing two distinct Ca\(^{2+}\) -binding domain types: PLC\(\gamma\) that has the C2 domain and an EF-hand (Essen \textit{et al.}, 1996).

The preference for calcium

The following question invariably arises: Why is Ca\(^{2+}\) so effective as a second messenger and why was this particular ion is selected through evolution for the important messenger function? Calcium is one of the four important alkali and alkaline earth metals in living systems, along with Mg\(^{2+}\), Na\(^{+}\), and K\(^{+}\), that might qualify for the same job. The calcium-modulated proteins must not only distinguish calcium from other ions, but also be able to bind calcium in presence of much higher concentrations of e.g. Mg\(^{2+}\) and Na\(^{+}\). In reality, the special combination of charge distribution and ionic radius of the calcium ion permits a high degree of selectivity, even though it has the same charge as Mg\(^{2+}\) and roughly the same radius as Na\(^{+}\). Experiments from model systems have shown that a divalent cation will bind to a specific anionic site with preference to a monovalent ion with equal diameter if the distance between the anionic ligands is not much greater than the diameter of the cation. On the other hand, if the distance between the anionic ligands is much larger, specificity is reversed and the monovalent cation is preferred (Blaustein, 1985)

How can proteins then distinguish between Ca\(^{2+}\) and Mg\(^{2+}\) which have the same overall charge? First of all, calcium is substantially larger than magnesium, i.e. given the same coordination number calcium is at least 0.23Å larger.
This means that the calcium ion has a larger surface area and thus prefers more ligands than does magnesium. Calcium shows larger variability with respect to its ligands, with the frequency of coordination number being $8 > 7 > 6 > 9$ (Einspahr and Bugg, 1981). Magnesium, on the other hand, due to its closed-shell p-orbital electronic configuration, prefers a strict octahedral (i.e. six-coordinate) arrangement of its ligands with distances between ion and ligands in the 2.0-2.1Å range (Martin, 1983). From a static and dynamic point of view, Mg$^{2+}$ forms tighter and more stable complexes than calcium. This means that exchange of magnesium from a protein can not be achieved at the same rapid pace as with calcium, in other words magnesium-modulated proteins would be less effective. Additionally, preference for calcium over magnesium is achieved by exploiting the principle that calcium binds stronger to oxygen ligands whereas magnesium binds stronger to nitrogen ligands (Martin, 1984). Within the cells, magnesium is generally associated with phosphates whereas calcium is bound by proteins. In order for these proteins to distinguish an increased calcium concentration in the presence of relatively high constant concentrations of Mg$^{2+}$ and K$^+$, they must have dissociation constants for calcium in the $10^{-6}$ range and affinity for Mg$^{2+}$ and K$^+$ at least $10^3$ and $10^5$ less, respectively. However, it has been speculated that many CaBPs have been designed to accommodate both calcium and magnesium in the same binding site but under different conditions (Strynadka, 1989).

**Three major Ca$^{2+}$-binding sensor families in plants**

To date, three major Ca$^{2+}$-binding sensor families have been found in plants. One of the most extensively characterized calcium sensor families is calmodulin (CaM); CaM is a highly conserved, well-characterized and ubiquitous calcium receptor in eukaryotes. It has four calcium-binding EF-hand motifs. The second major studied family is Ca$^{2+}$-dependent protein kinases (CDPKs), which have EF-hands and protein kinase domain also. The third major family is calcineurin-B-like (CBL) proteins, which are most recently found calcium sensors. Many of these
proteins bind Ca\(^{2+}\) using a helix-loop-helix structure termed the ‘EF hand’, which binds a single Ca\(^{2+}\) molecule with high affinity (Natalie et al., 1989). Frequently, pairs of EF hands interact through antiparallel β-sheets, which allows cooperatively in Ca\(^{2+}\) binding. When Ca\(^{2+}\) binds to [Ca\(^{2+}\)]\(_{\text{cyt}}\) sensors their structural and/or enzymatic properties change and their subsequent interactions with target proteins can alter solute transport and enzymatic activities, cytoskeletal orientation, protein phosphorylation cascades and gene expression. It is believed that these changes result in stress tolerance and/or a developmental switch. The form of the physiological response is determined not only by the [Ca\(^{2+}\)]\(_{\text{cyt}}\) perturbation itself, but also by the expression of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) sensors, their affinities for both Ca\(^{2+}\) and target proteins, and the abundance and activity of the target proteins. Since different cell types, and probably even individuals of the same cell type, have contrasting transcript, protein and enzyme profiles, a similar [Ca\(^{2+}\)]\(_{\text{cyt}}\) perturbation will result in individual responses, which may contribute to phenotypic plasticity (Gilroy and Trewavas, 2001).

**Calcium-binding proteins: CaM and CaM-related proteins**

CaM is found in the apoplast and in the cytosol, ER and nucleus of plant cells. Within the cytosol, the estimated CaM concentration is 5 to 40 μM (Zielinski, 1998). Calmodulin has been implicated in Ca\(^{2+}\)-dependent responses to light, gravity, mechanical stress, phytohormones, pathogens, osmotic stress, salinity, heavy metals, xenobiotics, anoxia, oxidative stress, heat shock and chilling (Zielinski, 1998; Snedden and Fromm, 2001; Reddy, 2001; Rudd and Franklin-Tong, 2001; Fasano et al., 2002). Calmodulin is a small (17 kDa), highly conserved, acidic protein with two globular domains each containing two EF hands connected by a flexible α-helical spacer (Zielinski, 1998; Reddy, 2001; Snedden and Fromm, 2001; Luan et al., 2002). A Ca\(^{2+}\)/CaM complex generally interacts with target proteins, although there are exceptions, such as the interaction of myosin-like proteins with CaM alone (Reddy, 2001). The binding of Ca\(^{2+}\) to CaM, which has a \(K_d\) between
10^{-7} and 10^{-6} \text{M}, exposes a hydrophobic surface on each globular domain. This enables the Ca^{2+}/CaM complex to wrap around its target protein and bind to its CaM-binding site with an affinity in the nanomolar range through non-specific van der waals interactions. The co-location of two EF hands in each globular domain allows Ca^{2+} to bind cooperatively, which ensures that CaM activation occurs over a narrow range of Ca^{2+} concentrations. The affinity of CaM for Ca^{2+} may be profoundly influenced by the presence of particular target proteins (Zielinski, 1998). Calmodulins bind to many different proteins implicated in diverse physiological processes including cation transport (including \([\text{Ca}^{2+}]_{\text{cyt}}\) homeostasis), cytoskeletal rearrangements and cell division, phytohormone and phospholipid signalling, disease resistance (including the oxidative burst) and stress tolerance (Zielinski, 1998; Reddy, 2001; Snedden and Fromm, 2001; Luan et al., 2002; Reddy et al., 2002). Calmodulins can also regulate gene expression by binding to specific transcription factors (Szymanski et al., 1996; Reddy et al., 2000; Yang and Poovaiah, 2000; Bouché et al., 2002).

Small gene families encode CaM isoforms in plants. Many species possess several CaM genes encoding identical proteins as well as other genes encoding divergent isoforms (Zielinski, 1998; Snedden and Fromm, 2001). It is thought that the presence of genes encoding identical CaMs may reflect a need for diverse tissue-specific, developmental or stress-induced expression, and the presence of genes encoding different CaM isoforms may reflect a requirement for specific interactions with target proteins (Reddy, 2001; Snedden and Fromm, 2001). Indeed, it has been suggested that particular CaM isoforms transduce specific environmental or developmental signals. Consistent with this hypothesis, it has been shown that environmental challenges rapidly up-regulate the expression of different CaM isoforms. For example, a subset of CaM genes is up-regulated by touch in various plants (Braam et al., 1997; Verma and Upadhyaya, 1998; Zielinski, 1998). Cold shock and wind stimuli lead to the expression of different CaM...
isoforms (van der Luit et al., 1999), and specific CaM genes are up-regulated in response to wounding or pathogens (Heo et al., 1999; Yamakawa et al., 2001), auxin or salinity (Botella and Arteca, 1994). Many CaM isoforms show tissue-specific and/or developmentally regulated expression (Gawienowski et al. 1993; Takezawa et al., 1995; Yang et al., 1998; Yamakawa et al., 2001; Duval et al., 2002), and CaM isoforms can differentially regulate target enzymes or have contrasting affinities for Ca\textsuperscript{2+} and CaM-binding peptides (Liao et al., 1996; Liu et al., 1998; Reddy et al., 1999; Köhler and Neuhaus, 2000; Lee et al., 2000; Duval et al., 2002; Zielinski, 2002). Calmodulins can also be post-translationally trimethylated, which influences both their stability and physiological activities (Zielinski, 1998). All these properties are consistent with [Ca\textsuperscript{2+}]\textsubscript{cyt} signatures producing unique biochemical and physiological consequences in cells expressing specific CaM isoforms and target proteins. Interestingly, target proteins themselves may have CaM-binding or non-binding isoforms (Snedden and Fromm, 2001), which presumably allows for [Ca\textsuperscript{2+}]\textsubscript{cyt}-dependent and [Ca\textsuperscript{2+}]\textsubscript{cyt}-independent regulatory cascades.

Plants also possess ‘CAM-like’ proteins. These have between one and six EF hands and a limited homology to CaM (defined arbitrarily as <75% identity with canonical CaM isoforms; Reddy, 2001; Snedden and Fromm, 2001; Luan et al., 2002; Zielinski, 2002). In Arabidopsis, they include: CaBP-22 (Ling and Zielinski, 1993), TCH2 and TCH3 (Braam et al., 1997), AtCP1 (Jang et al., 1998), centrins (Cordeiro et al., 1998), NADPH oxidases (Torres et al., 1998), homologues of the rice ABA-inducible EFA27 protein (Frandsen et al., 1996) and Ca\textsuperscript{2+}-binding protein phosphatases such as ABI1 and ABI2 (Leung et al., 1997). These proteins have been implicated in cellular responses to diverse environmental, developmental and pathological challenges.
Calcium-binding proteins: calcineurin B-like proteins

Calcineurin B-like proteins (CBL) possess three EF hands (Luan et al., 2002). In *Arabidopsis* there are at least ten *AtCBL* genes, including *AtSOS3* (*AtCBL4*), which encodes a Ca\(^{2+}\)-sensor protein involved in salt tolerance (Liu and Zhu, 1998; Luan et al., 2002; Xiong et al., 2002). Many CBLs have a conserved myristoylation site in their N-termini, which allows membrane association. It has been proposed that myristoylation of CBLs alters their cellular location and intracellular interactions (Luan et al., 2002). It is thought that particular CBLs transduce specific environmental or developmental signals. Consistent with this hypothesis is the induction of *AtCBL1* expression by drought, salinity, cold and wounding (Kudla et al., 1999; Piao et al., 2001) and the accumulation of *AtCBL1* and *AtCBL2* transcripts in leaves upon illumination (Nozawa et al., 2001). A family of SNF1-like serine/threonine protein kinases (*AtCIPK1* through *AtCIPK25*) has been identified as Ca\(^{2+}\)-dependent targets for CBLs (Shi et al., 1999; Halfter et al., 2000; Albrecht et al., 2001; Y. Guo et al., 2001; Luan et al., 2002). The CIPKs interact with CBLs through a unique 24-amino-acid domain termed the ‘NAF domain’ (Albrecht et al., 2001) and require divalent-cation cofactors for their activity (Luan et al., 2002). Both *AtCIPK1* and *AtCIPK2* require Mn\(^{2+}\) as a cofactor and *AtSOS2* (*AtCIPK24*) requires Mg\(^{2+}\). Each CBL protein may interact with several CIPKs. For example, *AtCBL1* interacts with numerous *AtCIPKs* (Shi et al., 1999) and *AtSOS3* interacts with *AtSOS2* (*AtCIPK24*) and at least seven other *AtCIPKs* (Halfter et al., 2000; Y. Guo et al., 2001). Conversely, certain *AtCIPKs* can interact with several *AtCBLs* (Shi et al., 1999; Kim et al., 2000; Albrecht et al., 2001; Y. Guo et al., 2001). Common interactions between *AtCBLs* and *AtCIPKs* may allow cross-talk between signalling cascades, whereas preferential associations between CBLs and CIPKs are thought to underpin specific signalling cascades. In this context it is noteworthy that the expression of *CIPKs* (in addition to *CBLs*) can be tissue specific and/or regulated by environmental stresses such as cold, drought, salinity,
wounding or nutrient starvation (Albrecht et al., 2001; Y. Guo et al., 2001; Kim et al., 2003).

**Calcium-binding proteins: calcium-dependent protein kinases**

The activity of many protein kinases can respond to \([\text{Ca}^{2+}]_{\text{cyt}}\) signals directly. These can be placed in one of four classes: \(\text{Ca}^{2+}\)-dependent protein kinases (CDPKs), CDPK-related proteins (CRKs), CaM-dependent protein kinases (CaMKs) and chimeric \(\text{Ca}^{2+}\)- and CaM-dependent protein kinases (CCaMKs). McAinsh and Hetherington (1998) proposed an interesting model for generating specific physiological responses to \([\text{Ca}^{2+}]_{\text{cyt}}\) perturbations through the action of \(\text{Ca}^{2+}\)-dependent and \(\text{Ca}^{2+}\)-independent protein kinases and phosphatases. In this model, specific \([\text{Ca}^{2+}]_{\text{cyt}}\) perturbations influence the degree of phosphorylation of a target protein leading to contrasting or graded responses.

The CDPKs are ubiquitous in plants. There are at least 34 genes encoding CDPKs in the *Arabidopsis* genome (Harmon et al., 2001; Cheng et al., 2002) and similar numbers in other plant species. They generally have four EF hands at their C-terminus that bind \(\text{Ca}^{2+}\) to activate their serine/threonine kinase activity. They act as monomers and many may be autoinhibited by autophosphorylation of a pseudosubstrate domain (Cheng et al., 2002). Different CDPKs have contrasting affinities for \(\text{Ca}^{2+}\) (Lee et al., 1998) and the binding of \(\text{Ca}^{2+}\) to some CDPKs is modulated by lipids, interactions with 14-3-3 proteins or phosphorylation (Reddy, 2001; Cheng et al., 2002; Sanders et al., 2002). No CDPKs appear to be integral membrane proteins, but many are associated with the cytoskeleton, nucleus, plasma membrane and ER (Reddy, 2001; Sanders et al., 2002). At least 24 *Arabidopsis* CDPKs can potentially undergo myristoylation and palmitoylation at their N-termini, which may facilitate their association with membranes (Cheng et al., 2002; Xiong et al., 2002).
The CDPKs are capable of converting $[\text{Ca}^{2+}]_{\text{cyt}}$ signals into biochemical and genetic consequences through the phosphorylation of diverse target proteins including membrane solute transporters (including the Ca$^{2+}$-ATPase, AtACA2), ion and water channels, NADPH oxidases, enzymes involved in carbon and nitrogen metabolism, cytoskeletal proteins, proteases and DNA-binding proteins (Reddy, 2001; Rudd and Franklin-Tong, 2001; Cheng et al., 2002; Sanders et al., 2002). They are implicated in pollen development, control of the cell cycle, phytohormone signal transduction, light-regulated gene expression, gravitropism, thigmotropism, nodulation, cold acclimation, salinity tolerance, drought tolerance and responses to pathogens (Sheen, 1996; Saijo et al., 2000; Anil and Sankara Rao, 2001; Reddy, 2001; Romeis et al., 2001; Cheng et al., 2002; Xiong et al., 2002; Lee et al., 2003). It is thought that the possession of many CDPKs with contrasting Ca$^{2+}$ affinities and target proteins allows plant cells to respond appropriately to specific $[\text{Ca}^{2+}]_{\text{cyt}}$ perturbations (Anil and Sankara Rao, 2001; Cheng et al., 2002; Sanders et al., 2002). In various plant species specific CDPKs are induced by cold, drought, salinity, anoxia, mechanical stress, wounding and pathogen elicitors (Urao et al., 1994; Breviario et al., 1995; Monroy and Dhinsa, 1995; Botella et al., 1996; Tähthiharju et al., 1997; Yoon et al., 1999; Saijo et al., 2000; Anil and Sankara Rao, 2001; Romeis et al., 2001; Chico et al., 2002; Lee et al., 2003). In addition, the activities of CDPKs are affected by post-translational modifications during development (Anil et al., 2000) or in response to environmental challenges such as wounding or pathogens (Romeis et al., 2001).

Other protein kinases responding to $[\text{Ca}^{2+}]_{\text{cyt}}$ are less well characterized than the CDPKs. There are at least seven CRKs in the *Arabidopsis* genome that are structurally similar to CDPKs, but have degenerate or truncated EF hands that may not be able to bind Ca$^{2+}$ (Reddy, 2001). Orthologues of these are present in many plant species. Several CaMKs have also been cloned from *Arabidopsis* and other plants (Zhang and Lu, 2003). Their kinase activity is activated by CaM-
dependent autophosphorylation and their catalytic activity is also modulated by CaM. They are expressed highly in rapidly growing cells and tissues of the root and flower (Zhang and Lu, 2003). Chimeric CCaMKs are expressed in the anthers of several plant species, including lily and tobacco (Liu et al., 1998), but none have been identified in *Arabidopsis* (Zhang and Lu, 2003). The CCaMKs possess a CaM-binding domain and three EF hands. They require only Ca\(^{2+}\) for autophosphorylation, but Ca\(^{2+}\) and CaM for substrate phosphorylation. Liu et al. (1998) demonstrated that different CaM isoforms have contrasting effects on substrate phosphorylation by lily and tobacco CCaMKs, suggesting that these kinases could respond to specific environmental or developmental challenges.

**EF-hand-containing proteins in *Arabidopsis***

In plants, calcium (Ca\(^{2+}\)) has emerged as an important messenger mediating the action of many hormonal and environmental signals, including biotic and abiotic stresses. Many different signals raise cytosolic calcium concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)), which in turn is thought to regulate cellular and developmental processes via Ca\(^{2+}\) -binding proteins.

**Identification of EF-hand-containing proteins**

Day *et al.*, (2003) to identify EF- hand-containing proteins in *Arabidopsis*, Each protein sequences analyzed in the presence of an EF- hand motif and other domin(s) using interProScan. they was chosen InterProScan, because it integrates SWISS-PORT, PROSITE, PRINTS, Pfam, ProDom, SMART and TIGRFAMs programs into a single comprehensive format. Therefore, scanning one site is the equivalents of scanning seven databases that use different approaches (Apweiler *et al.*, 2001).
The Interpro Domain Table at MAtdB listed 219 proteins as having EF-hands. Eighteen sequences did not have EF-hands identifiable by InterProScan. So they also did sequence-similarity searches using three different EF-hand proteins that have been characterized in Arabidopsis. The nucleotide and protein sequences of Arabidopsis CAM4, a protein containing four EF-hands, were used to do BLAST searches (TblastN, BlastP) against the Arabidopsis genome at MAtdB (Schoof et al., 2002). They used the protein sequences of a Ca2+-dependent protein kinase (CPK1) and a small protein with one EF-hand domain (At2g46600). Proteins showing similarity to these proteins were checked for the presence of EF-hands using InterProScan as above. They also searched the literature for reports of EF-hand-containing proteins in Arabidopsis that had been identified by various experimental approaches. Additional EF-hand proteins were identified from this search.

Together, these searches resulted in identifying a total of 250 possible EF-hand containing proteins. However, only 47 have been confirmed their calcium-binding but the rest were yet to be verified for Ca2+-binding. Furthermore, each of these putative calcium-binding proteins has a variety of protein functional domains that may be useful in determining their biological functions. The InterPro domain table also lists the EF-hand-containing proteins for Saccharomyces cerevisiae (29), Caenorhabditis elegans (139) and Drosophila melanogaster (132).

**EF-hand calcium binding proteins**

The super family of EF-hand helix-loop-helix Ca2+ -binding proteins (Persechini et al., 1989) represents a very large group of proteins sharing a common structural motif, which consists of a Ca2+-binding loop flanked by 2a-helices. This motifs allows rapid and reversible binding of Ca2+, with dissociation constants in the Ca2+ physiological range (0.01-10mM). The EF-Hand
super family can be divided in 39 subfamilies, comprising at the present times more than 250 proteins.

EF-hand CaBP are found in all eukaryotes cells. Generally they are localized in the cytosol (in few cases associated with membranes), although some are found within organelles and nucleus. In multicellular organisms, some are present ubiquitously (like calmodulin) or (more) restricted to specific cells (like recoverin in photoreceptors or troponin-C in skeletal and cardiac muscle).

The EF-hand Ca\(^{2+}\)-binding site

The canonical EF-hand site consists of a a-helix (about 10 residues), a loop containing a b- pleated sheet (12 residues), and a second a-helix (11 residues) oriented almost perpendicularly with respect to the first.

The Ca\(^{2+}\) is coordinated, in the loop, by 7 oxygen ligands, which are provided (for 5) by (acidic) side chains, (for 1) by a carbonyl group of the peptide backbone, (for 1) by a bridging water molecule (Figure. 2a). The spatial configuration of the ligands around the calcium is pentagonal bipyramidal. The six residues involved in the binding are in relative positions 10, 12, 14, 16, 18, 21; these residues are named X, Y, Z, -Y, -X, -Z. From the sequence of the different EF-hand CaBP a consensus sequence for the EF-hand motif (Figure. 2c) (Kretsinger, 1987) has been derived. The calcium coordinating residues X(10), Y(12), Z(14), -Z(21) provide oxygen directly with their side-chains for the coordination: Asp, Glu are the most frequent, but Asn, Gln, Ser, Thr are also found. Position X(10), Y(12), and -Z(21) are the most conserved. The invariant Glu or Asp at position 21 provides 2 oxygen for liganding Ca\(^{2+}\) (bidentate ligand). The -Y(16) residue just provide a carbonyl from the peptide backbone to coordinate the Ca\(^{2+}\) (therefore it can harbor almost any side-chain). The -X(18) residue frequently...
Figure: 2

A. 3D structure of an EF-hand from the parvalbumin visualized using the ViSP software created by Edouard deCastro on a Silicon Graphics IRIX 4D/35 computer;

B. Anthropomorphic representation of an EF-hand;

C. Consensus sequence of the EF-hand domain.
harbors an oxygen bearing side chain that helps to stabilize the coordinating H2O molecule.

The three-dimensional structure of the EF-hand site can be represented by the right hand, with the index finger representing the 1st helix, the bent middle finger the loop, and the thumb the 2nd helix (Figure 2b). Therefore the structure as been named "Hand". The designation EF derives from the C-terminal E and F helices in parvalbumin, the first EF-hand CaBP that was crystallized. Parvalbumin (Kretsinger and Nockolds, 1973) contains 6 a-helices, which were called A, B, C, D, E, F starting from N-terminus. Two Ca2+ ions were found in the loops joining helices C to D and E to F.

Although simple EF hand polypeptide have only a low affinity for Ca2+, their presence in pairs is associated with high affinity binding. The EF-hand loop contains a B-pleated sheet, which allows the pairing of 2 EF-hands into a tandem domain (2 sites domain) (Strynadka and James, 1989). This tandem domain is a basic feature common to all EF-hand Ca2+-binding proteins. It seems to be important for protein folding as well as for the functional properties of individual Ca2+ -binding sites.

**Calcium-binding proteins: proteins without EF hands**

Several proteins lacking EF hands are also capable of binding Ca2+ (Reddy, 2001; Anil and Sankara Rao, 2001). For example, the activity of phospholipase-D (PLD), which cleaves membrane phospholipids into a soluble head group and phosphatidic acid, is regulated by [Ca2+]cyt through a Ca2+/phospholipid binding site termed the ‘C2 domain’ (Wang, 2001). Phospholipase D 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 (Ritchie et al., 2002). 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\(_3\) and diacylglycerol, phospholipase A\(_2\) and diacylglycerol kinase, both of which are regulated by CaM. It has therefore been suggested that [Ca\(^{2+}\)]\(_{cyt}\) signalling cascades might coordinate the activities of these diverse enzymes to effect specific responses to contrasting environmental or developmental stimuli (Wang, 2001).

In annexins, Ca\(^{2+}\) is bound by the ‘endonexin fold’, which contains a characteristic GXGT-[38]-(D/E) motif (Delmer and Potikha, 1997). This often appears only in the first quarter of plant annexins, although animal annexins generally possess four such motifs. The binding of Ca\(^{2+}\) to annexins enables them to associate with membranes to form cation-channels (Hofmann et al., 2000; White et al., 2002a). In plants, annexins are encoded by small gene families. Seven annexin genes (AnnAt1-7) have been identified in Arabidopsis (Clark et al., 2001; White et al., 2002a). Most annexin genes are expressed throughout the plant, but annexins are especially abundant in highly secretory cell types, where they are located at the cell periphery and may have a role in membrane fusion, membrane trafficking and/or secretion (Clark et al., 2001). The increased expression of certain annexins upon specific developmental or environmental challenges has linked them with Ca\(^{2+}\) signals during root nodulation, pathogen attack, ABA responses, fruit ripening and cold acclimation (White et al., 2002a).

Other Ca\(^{2+}\)-binding proteins include calreticulin, calsequestrin, calnexin and BiP, which sequester Ca\(^{2+}\) in the ER. These proteins are implicated in Ca\(^{2+}\) homeostasis, protein folding and post-translational modifications (Crofts and Denecke, 1998; Michalak et al., 1998). The expression of calreticulin is increased in reproductive tissues. Calreticulin possesses two Ca\(^{2+}\)-binding domains. The first is a proline-rich, low-capacity/high-affinity Ca\(^{2+}\)-binding domain (\(K_d =1 \mu M; 1 \text{ mol Ca}^{2+} \text{ mol protein}^{-1}\)) that contains a highly conserved stretch of amino acids...
The second is a high-capacity/low-affinity Ca\textsuperscript{2+}-binding domain \((K_d = 2 \text{ mM}; 25 \text{ mol Ca}^{2+}/\text{ mol protein})\) comprising acidic amino acid residues (generally glutamate and aspartate). Both calreticulin and BiP, and a Ca\textsuperscript{2+}-binding protein (PCP) expressed in Brassica pistils and implicated in pollen-pistil interactions (Furuyama and Dzelzkalns, 1999) also possess high-capacity/low-affinity Ca\textsuperscript{2+}-binding domains. Calnexin possesses a proline-rich low-capacity/high-affinity Ca\textsuperscript{2+}-binding domain.

**Number of EF hands**

The number of EF-hands in each protein varied from one to six. Most EF-hand proteins have pairs of EF-hands, which facilitate binding of Ca\textsuperscript{2+} (Nakayama et al., 2000). There are a large number of proteins with an odd number of EF-hand motifs (1, 3 or 5). The proteins with an odd number of EF-hand domains may function as homo- or heterodimers, they may bind Ca\textsuperscript{2+} in a weaker manner, there may be another 'cryptic' Ca\textsuperscript{2+}-binding motif that is not identifiable, but is functional, or they may not bind Ca\textsuperscript{2+} at all. Many of the proteins containing a single EF-hand motif were identified by only one prediction program and could be false positives. Examples of these possibilities can be seen in EF-hand proteins that have been isolated and characterized previously. The K\textsuperscript{+} channel protein (KCO1) has one identifiable EF-hand but another region within the protein also shows similarity to an EF-hand. Although Ca\textsuperscript{2+}-binding of KCO1 was not tested, the activity of the channel was shown to be Ca\textsuperscript{2+}-dependent (Czempinski et al., 1997). AtPLC1, one of a small family of phosphatidylinositol-specific phospholipase Cs (PLCs), has a putative EF-hand but Ca\textsuperscript{2+} binding was not evaluated (Hirayama et al., 1995). No other AtPLC has an EF-hand domain but the amino-terminal sequences of several other family members have two sets of a helices that may correspond to EF-hand domains (Otterhag et al., 2001). The putative EF-hand loop of AtPLC1 lies between two of the helices. The actin-binding activity of most fimbrins is inhibited by
Ca\(^{2+}\) (Kovar et al., 2000) AtFIM2 was shown to be Ca\(^{2+}\) independent, suggesting this single-EF-hand protein does not bind Ca\(^{2+}\) (McCurdy et al., 1998; Kovar et al., 2000).

**Phylogenetic analysis of EF-hand-containing proteins**

Day et al. (2003) Phylogenetic analysis was carried out by PAUP 4.08a using a heuristic search method. A consensus tree was generated from all saved trees. This tree was used to identify groups of EF-hand proteins and closely related proteins. Five major groups of proteins could be identified. Figure 3 shows the overall tree with a few representative members of each group. A sixth group includes members that did not fall into the other five groups.

**Group I proteins**

None of the proteins in group I has been reported in the literature. Some of them contain domains that give clues to their function, including elongation factors, DNA-, protein- or ATP/GTP-binding proteins (Lewit-Bentley and Rety, 2000).

**Group II proteins**

Group II includes KCO1, AtPLC1 and the two fimbrins have been reported in the literature. Two other proteins show similarity to KCO1 and may also be Ca\(^{2+}\)-regulated K\(^+\) channels. A family of phosphatidylinositol-specific phospholipase Cs have been isolated, but only one of them, AtPLC1, has an EF-hand domain (Otterhag et al., 2001; Hirayama et al., 1995). AtPLC1, a protein isolated as a dehydration and salt stress-induced gene, was able to hydrolyze phosphatidylinositol-4,5-bisphosphate and the activity was completely dependent on Ca\(^{2+}\) (Hirayama et al., 1995). AtFIM1 was identified as an EF-hand-containing protein however, Kovar et al. (2000) found that AtFIM1 was Ca\(^{2+}\)-independent and...
Figure: 3

Phylogenetic tree showing the overall relatedness of the EF-hand proteins. All EF-hand proteins were aligned using MEGALIGN (DNAsar) and analyzed using a heuristic method in PAUP 4.08a. Numbers represent the number of times the branch appeared in 100 saved trees. The tree was reduced by hand to show a few representative proteins for each major group.
so this may be a non-functional EF-hand as pointed out by McCurdy and Kim (1998).

**Group III proteins**

CBL/SOS3S fall into group III. The first CBL/SOS3 was isolated as a protein involved in salt stress (SOS3) and as a calcineurin B-like protein (CBL1) (Kudla et al., 1999; Liu and Zhu, 1998). Ten CBL/SOS3S have been identified (Luan et al., 2002). Expression of CBL4 is induced by drought, cold and wounding stress. In animals, calcineurin is a heterodimer composed of a regulatory B subunit and a protein phosphatase catalytic A subunit. CBL/SOS3S show similarity to the B subunit. SOS3 was shown to interact with a protein kinase (Halfter et al., 2000) and a family of interacting protein kinases has been identified Kim KN et al., 2000; Albrecht et al., 2001) AtCP1, which contains three EF-hands is another NaCl-stress-induced protein that has been shown to bind Ca$^{2+}$ (Jang et al., 1998). A bean homolog of AtCP1 has been shown to be associated with the hypersensitive response (Jakobek et al., 1999). A subgroup of proteins identified in this search show similarity to the protein phosphatase 2A regulatory B subunit. One of these, At5g44090 (AF165429), was reported in the literature (Hendershot et al., 1999) they have one EF-hand domain but contain no other identifiable domains. One other protein of interest in this group, KIC (KCBP-interacting CCD-1-like protein), was identified as a protein that interacts with KCBP (kinesin-like calmodulin-binding protein), a protein known to interact with and be regulated by Ca$^{2+}$/calmodulin (Reddy et al., 1996; Reddy et al., 1999). KIC has only one EF-hand and is similar to a wheat Ca$^{2+}$-binding protein (CCD-1) Takezawa (2000).

**Group IV proteins**

Group IV contains the calmodulins (CAMs) and closely related proteins such as CaBP-22, centrin and the TCH gene proteins. CAMs are highly conserved small-molecular-weight acidic proteins of 148 amino acids (listed as 149, the
starting Met is cleaved following translation). The four EF-hands (two pairs connected by a central helix) bind four molecules of Ca$^{2+}$ (Nakayama et al., 2000; Lewit-Bentley and Rety, 2000). Binding of Ca$^{2+}$ to CAM results in a conformational change which then allows CAM to interact with target proteins to modulate their activity or function (Reddy, 2000; Knight, 2000; O’Neil and DeGrado, 1990). Nine *Arabidopsis* CAMs have been reported in the literature (Perera and Zielinski, 1992; Ling, 1991; Gawienowski, et al., 1993; Zielinski, 2002; Braam and Davis, 1990).

Expression studies of the *Arabidopsis* CAM genes show that they are differentially expressed in different tissues and circumstances. For CAM1, CAM2, and CAM3, CAM1 was the only one expressed in roots and CAM3 could not be detected in floral stocks; CAM1, CAM2, and CAM3 are inducible by touch stimulation but at different levels and with different kinetics (Perera and Zielinski, 1992). CAM4, CAM5, and CAM6 were all expressed in leaves, but only CAM4 and CAM5 were detected in siliques (Gawienowski et al., 1993). Different *Arabidopsis* CAM isoforms also differ in their affinity for the same protein (Reddy et al., 1999; Liao et al., 1996).

Two proteins induced by touch, rain, wind, wounding, and darkness, TCH2 and TCH3, are also in group IV and are related to the CAMs. TCH2 has 161 amino acids with four EF-hands and TCH3 has 324 amino acids with six EF-hands. Another CAM-like protein, CaBP-22, is closely related to the conventional Cams. It has 191 amino acids, 66% amino-acid sequence identity with CAM (79% in the EF-hand domains) and has been shown to bind Ca$^{2+}$ (Ling and Zielinski, 1993). Centrins are a little more distantly related to CAMs. An *Arabidopsis* centrin gene (ap3.3a) was isolated as a gene rapidly induced after pathogen inoculation (Cordeiro et al., 1998). One other EF-hand protein is 65% similar to centrin, suggesting there are two centrin genes in *Arabidopsis*. 
**Group V proteins**

The 34 CPKs and three CPK-related protein kinases (CRKs) make up almost all of group V. CPKs are serine/threonine protein kinases with a CAM-like domain (CLD) usually containing four EF-hands (with two exceptions). These kinases have been called CDPKs (Harmon et al., 2000; Hrabak et al., 1996). Three of the eight CRKs in *Arabidopsis* have one EF-hand domain. However, sequence alignment of the EF-hand regions of CPKs and CRKs revealed that CRKs contain degenerate EF-hand motifs. CPKs are present only in plants and some protozoans. The plantsP database (http://plantsP.sdsc.edu) reports that most of the CPKs and CRKs contain transmembrane and N-myristoylation domains. TargetP predicts that some CPKs and CRKs are targeted to the chloroplast or mitochondria TargetP v1.01 (http://www.cbs.dtu.dk/services/TargetP/). The cellular localization for most of these protein kinases needs to be confirmed experimentally.

CRKs have similar domain organization as compared to CPKs. Most of the CPKs contain fatty-acylation sites, including those for myristoylation and palmitoylation, which seem to be necessary for targeting to membranes and for protein-protein interactions (Hrabak, 2000). The protein kinase domain in CRKs shows strong sequence similarity to the kinase domain in CPKs, but the auto inhibitory and CLDs in CRKs show weak sequence similarity to the corresponding domains in CPKs. CPKs have basal activities in the absence of Ca$^{2+}$ as a result of the presence of the autoinhibitory region. Ca$^{2+}$ binds the EF-hands of the CLD, which results in intramolecular rearrangement and relief of autoinhibition (Harmon et al., 2000; Weljie et al., 2000).

Eight CPK isoforms have been shown to be activated by Ca$^{2+}$ (Hrabak, et al., 1996; Hong et al., 1996; Urao, 1994 a, 1994b). The presence of multiple isoforms of CPKs in the *Arabidopsis* genome implies that they may be involved in specific Ca$^{2+}$-
signaling networks, may respond differentially to changes in oscillation, frequency, magnitude and duration of Ca\(^{2+}\) signal, or may have temporal and spatial patterns of expression and localization. Little is known about the function and substrates for CPKs in Arabidopsis. CPK1 is known to interact with 14-3-3 proteins (Camoni et al., 1998) and is involved in the inactivation of a Ca\(^{2+}\) pump (Hwang et al., 2000). Expression of CPK10 and 11 is inducible by cold and drought (Urao, et al., 1994; Sheen, 1996; Tahtiharju et al., 1997). An Arabidopsis CPK phosphorylates tonoplast intrinsic protein, α-TIP, a putative water-channel protein (Johnson and Chrispeels, 1992). Substrates of CPKs in other plants have been identified and can be used to deduce the function of homologs in Arabidopsis.

**Group VI**

The remaining proteins that do not fall into one of the other five groups. It includes the respiratory burst oxidase homology proteins (Rbohs) ABI1, GDH2, and TPC1. Torres et al. and Keller et al. (1998) isolated Arabidopsis homologs (Rbohs) to the gp91\(^{phox}\) subunit of the neutrophil NADPH oxidase, which generates a similar oxidative burst in neutrophils. Six Rbohs (A-F) were identified in the Arabidopsis genome and their EF-hands were shown to bind Ca\(^{2+}\). Both Leung et al. and Meyer et al. (1994) isolated the ABI1 gene. ABI1 is similar to serine/threonine phosphatase 2C, which in animals is Mg\(^{2+}\) or Mn\(^{2+}\) but not Ca\(^{2+}\)-dependent. ABI1 is induced by abscisic acid and was shown to regulate stomatal aperture in leaves and mitotic activity in root meristems (Leung et al., 1994; Meyer et al., 1994). A second ABI gene (ABI2) was isolated using ABI1 as a probe (Leung, 1997). The protein encoded by the cDNA had an eight-residue insertion in the EF-hand domain that does not conform to the EF-hand signature. A similar situation holds for glutamate dehydrogenases. Two genes were isolated; one coded for a
dehydrogenase with an EF-hand (GDH2) and the other one without (GDH1) (Turano et al., 1997) Studies by Furuichi et al., (2001) indicate that TPC1 is a two-pore channel that mediates Ca\(^{2+}\) influx. It has two EF-hand-like motifs located in a hydrophilic domain that connects the two transmembrane regions containing the pores. Ca\(^{2+}\) binding was not shown experimentally for ABI1, GDH2, or TPC1. Another three proteins in this group have a domain present in a small GTPase protein.

**Analysis of the small GTPase gene superfamily of Arabidopsis**

Small GTP-binding proteins are molecular switches that are “activated” by GTP and “inactivated” by the hydrolysis of GTP to GDP. The resulting cycles of binding and hydrolysis of GTP by small GTP-binding proteins represents a ubiquitous regulatory mechanism in eukaryotic cells. Members of this class of proteins are among the largest families of signaling proteins in eukaryotic cells. Their importance in cellular signaling processes is underscored by their conservation throughout evolution of eukaryotic organisms and by the presence of homologs that perform related functions in cells of yeasts, humans and plants. Although the GTP-hydrolysis “core” of this class of regulatory molecules is highly conserved, the surrounding domains are highly variable and undergo conformational changes as these proteins switch from GTP-associated to GDP-associated states. Eukaryotes have harnessed this diversity of protein conformations, linked to the nucleotide-associated state of the GTP-binding domain, to regulate a myriad of cellular processes (Takai et al., 2001). Small GTP-binding proteins are involved in regulation of diverse eukaryotic cellular processes, such as cell proliferation, cytoskeletal assembly and organization, and intracellular membrane trafficking (Barbacid, 1987; Boguski and McCormick, 1993; Takai et al., 2001). Physiological control of these GTPase “switches” occurs through association of the GTPase with accessory proteins, termed guanine nucleotide exchange factors (GEFs), that catalyze the conversion of the small GTP-binding
protein to their GTP bound “active” conformation. In their “active” state, small GTPases interact with various downstream “effector” proteins that perform the diverse cellular functions controlled by this class of regulatory molecules. Inactivation occurs through either the intrinsic ability of the small GTP-binding protein to hydrolyze GTP to GDP_Pi, or through association with another set of accessory proteins, GTPase-activating proteins (GAPs), which stimulate this hydrolytic activity.

Structural and functional similarities between different members of this large superfamily has led to establishment of five distinct families: Ras, Rab, Rho, Arf, and Ran (Kahn et al., 1992). Ras GTPases regulate cell proliferation in yeast and mammalian systems. Members of the Rho GTPase family control actin reorganization and signal transduction pathways associated with MAP kinases. The Rab and Arf GTPase families function in distinct steps of membrane trafficking, whereas Ras-related nuclear protein (Ran) GTPases regulate transport of proteins and RNA across the nuclear envelope. Individual members of these families share higher overall sequence conservation with one another than with any other small GTPase families. Analysis of the genomes of *Saccharomyces cerevisiae*, fruitfly (*Drosophila melanogaster*), *Caenorhabditis elegans*, Arabidopsis, and human (*Homo sapiens*) has underscored the conservation of these classes of regulatory molecules and has provided interesting glimpses into the ways in which the small GTPases of these families have evolved and proliferated. In *Arabidopsis* 93 small GTP binding proteins were identified further these GTPases classified within four of the five small GTPase families: with 57 Rab GTPases; 21 Arf GTPases; 11 Rho GTPases; and 4 Ran GTPases. *Arabidopsis* does not contain any Ras GTPases that can be identified based on phylogenetic analysis, perhaps reflecting unique mechanisms for control of cell signaling during development in plants (Meyerowitz, 1999; 2002).
GTP-binding proteins (G-proteins) are well known molecular switches in signal transduction pathways which, through their interaction with signal transducing receptors, can turn the receptor ‘on’ (GTP-binding) or ‘off’ (GDP-binding). G-proteins act on various cellular activities, such as cell proliferation, cytoskeletal organization, intercellular membrane trafficking, and stress resistance (Zheng and Yang 2000). Two major classes of signaling G-proteins have been found to date, one is the heterotrimeric G-proteins and the other is the Ras superfamily of monomeric small GTPases, which consists of five subfamilies—RAS, RHO, RAB/YPT, ARF, and RAN. Among these subfamilies, only the RAS and RHO GTPases have been clearly shown to transmit extracellular signals. In plants, the RHO subfamily of GTPases (termed Rop, for ‘Rho of plants’) comprises small GTPases that are related to signal transduction (Zheng and Yang 2000). Rop GTPases control a wide range of cellular processes, most of which are linked to the regulation of the cytoskeleton, which probably reflects the conserved function of ancestral Rho GTPases, and also regulate plant responses to several major hormones, such as ABA, auxin and brassinosteroids (Burridge and Wennerberg 2004; Li et al. 2001).

**Rop is a plant-specific subfamily of RHO GTPases**

The Rho-family proteins are defined by the presence of a Rho-type GTPase-like domain. A structural feature that distinguishes the Rho proteins from other small GTPases is the so-called Rho insert domain located between the fifth b strand and the fourth a helix in the small GTPase domain (Valencia et al., 1991). Most typical Rho proteins are small (190-250 residues) and consist only of the GTPase domain and short N-and C-terminal extensions. However, some of the more atypical family members contain additional well-defined domains and can be >700 amino acids long. Within their GTPase domains, they share approximately 30% amino acid identity with the Ras proteins and 40-95% identity within the family.
Since the cloning of the first plant cDNA encoding a RHO-related GTPase (Rho1Ps) from pea in 1993 (Yang and Watson, 1993), a large number of Rho1Ps-like GTPases have been identified from various plants including mosses and higher plants (36 available in the database) (Yang and Watson, 1993; Delmer et al., 1995; Winge et al., 1997; Li et al., 1998). All but one of the 36 genes fall into a unique RHO subfamily termed Rop (RHO-related proteins from plants) (Li et al., 1998). Eleven Arabidopsis Rop genes have been identified to date (Li et al., 1998). Rops share >70% amino acid identity with each other and 45–64% identity with other members of the RHO family. Due to a slightly higher overall homology with the Rac subfamily of RHO GTPases, plant RHO related GTPases have often been named Racs in the literature (Delmer et al., 1995; Winge et al., 1997; Kawasaki et al., 1999; Kost et al., 1999; Potikha et al., 1999). However, phylogenetic analysis of RHO GTPases from three representative species, yeast, man and Arabidopsis and sequence comparison between Arabidopsis Rop and human Rho, Rac and Cdc42 clearly suggests that Rop is distinct from the three subfamilies of RHO GTPases from animals. Importantly, Rop is specific to plants, and plants apparently do not possess Cdc42, Rac, and Rho.

Rop is distinct from other RHO GTPases in several aspects. The highly conserved effector domain (domain II) contains several amino acid residues unique to Rop. All Rops contain two putative serine/threonine phosphorylation sites, SYR and SSK (with the exception of Rop6At, which has SAK as Rho). In addition, there are two other putative phosphorylation sites unique to specific Rop subgroups: SNK for Arac7, Arac8 and Arac10, and SKK for Rop1At, Rop3At, Rop5At, and Rop6At. These potential phosphorylation sites might be targets of RLKs, which have been suggested to associate with Rop (Trotochaud et al., 1999).

Rop can be further divided into at least four groups according to both overall sequence similarity and the variable region (domain VII) at the C-terminus.
Most Rops contain the geranylgeranylation motif CAAL (C, cysteine; L, leucine; A, aliphatic amino acids), the target for geranylgeranyl transferase II (GGTase II). The isoprenyl moiety covalently attached to the cysteine residue probably allows Rop to be anchored to membranes (Lin et al., 1996; Kost et al., 1999; Li et al., 1999; Rodriguez-Concepcion et al., 1999). In addition, most Rops (including groups III and IV) contain a CAAL-proximal polybasic region that presumably mediates the localization of Rop to specific membrane systems. Interestingly, the Rop members (group II) contained either the farnesylation motif CAAX or the geranylgeranylation motif CXX for GGTase II. These Rops contain some extra amino acid residues in the variable region due to the presence of an additional exon right next to the CAAX box. This is quite unique to the plant Rop, because intron numbers and positions are normally conserved among different small GTPases. Interestingly, the cysteine residues in the CAAX of maize Rop1 and Rop6 (group II) are not important for plasma membrane localization, suggesting either that these proteins are not isoprenylated or that isoprenylation is not critical for their membrane targeting (John Fowler, personal communication). In contrast, two internal cysteine residues in the variable region (domain VII) of these Rops have been shown to be critical for Plasma Membrane (PM) localization. Corresponding cysteine residues are also present in other members of this group. This suggests the existence of a novel PM targeting signal for this group of Rop GTPases.

Given the plant specificity and the large size of the gene family, one might speculate that Rop may serve as a ubiquitous switch in plant signal transduction analogous to heterotrimeric G proteins and RAS in animals. The large gene family and possible functional redundancy among family members present a potential problem for determining the function of Rop in plants, because loss-of-function mutations for a given Rop gene most likely do not have a phenotype. This appears to be the case because no Rop genes have been identified through forward genetic
approaches. However, the unique regulatory feature of GTPases, i.e., cycling between GDP- and GTP-bound forms, allows the generation of gain-of-function point mutations. These mutations have been extremely useful for functional studies of RHO and RAS GTPases in animals and yeast and have recently been proven to be useful for understanding the function of Rop in plants as well (Kawasaki et al., 1999; Kost et al., 1999a; Li et al., 1999; Potikha et al., 1999). Recent studies using this approach complemented with other techniques, such as injection of neutralizing antibody and over expression of sense and antisense genes, have implicated Rop in many pathways important for development and environmental responses in plants.

**Rop provides a potential link between signaling to the actin cytoskeleton and cell morphogenesis**

The actin cytoskeleton has long been thought to play an important role in plant cell morphogenesis and cell growth (Kost et al., 1999b). This view has been strengthened by several recent studies in trichomes and pollen tubes (Gibbon et al., 1999; Kost et al., 1999a; Mathur et al., 1999; Szymanski et al., 1999). However, the mechanism that controls plant actin organization and dynamics is poorly understood. Because RHO-family GTPases are conserved regulators that link extracellular signals to the organization of the actin cytoskeleton in yeast, *Dictyostelium*, and animals, it was speculated that Rop may also have a similar role in the regulation of the plant actin cytoskeleton. Indeed, alteration of F-actin in pollen tubes expressing CA-rop5At or DN-rop5At mutants has been reported (Kost et al., 1999a). The formation of extensive spiral cortical actin cables is associated with the expression of CA-rop5At, whereas DN-rop5At appears to cause reduced F-actin bundling in pollen tubes. However, it was suggested that these changes in actin organization are unlikely to account for the dramatic phenotypes induced by these mutants (Kost et al., 1999a). It remains to be
determined whether the changes in F-actin are directly due to alteration of Rop signaling or indirectly from morphological changes induced by Rop mutants.

Further support for the role of Rop in the regulation of actin organization in plant cells came from the studies of Rop2At in vegetative cells. Expression of rop2At dominant mutants under the CaMV 35S promoter caused changes in cell morphology similar to that induced by rop1Atmutants in pollen tubes. CA-rop2At induced isotropic cell expansion, whereas DN-rop2At inhibited cell expansion in Arabidopsis leaves. However, changes in the actin cytoskeleton resulting from the expression of these mutants seem to be different from those observed in pollen tubes by Chua's group (Kost et al., 1999a). In CA-rop2At cells, actin bundles become sub cortical, compared to cortical localization of these actin bundles in wild type cells. In DN-rop2At cells, however, the amount of fine cortical F-actin is drastically reduced, but thick actin cables do not seem to be affected. Although their significance in cell morphogenesis is not clear, these changes most likely are the direct effect caused by changes in Rop signaling activity. Rop signaling may be a general mechanism that controls actin-mediated plant cell morphogenesis.

**Rop in plant defense responses**

In mammalian cells, a multi-subunit plasma membrane NADPH oxidase is responsible for the generation of H2O2 in response to microbial stimuli. One key regulatory subunit is Rac2, which interacts with the p67phox regulatory subunit and is assembled into the catalytic subunit gp91phox together with the other regulatory subunit p47phox. Because of a crucial role for H2O2 in plant defense responses and programmed cell death and the existence of PM NADPH oxidase in plant cells, much attention has been devoted to the identification of a plant equivalent to the mammalian Rac2. It was shown that a Rac2-specific antibody detected a 21 kDa tomato protein that could be translocated to microsomal
membranes in response to elicitor treatments (Xing et al., 1997). Because plants apparently do not have Rac orthologues, this Rac2 antibody reactive protein, if a GTPase, most likely is a Rop. Indeed, recent studies show that constitutively active forms of the rice Rop OsRac1 constitutively activate H$_2$O$_2$ production (Kawasaki et al., 1999). CA-Osracl also induces spontaneous programmed cell death in rice leaves, whereas DN-Osracl inhibits H$_2$O$_2$ production and lesion formation induced by pathogen infection.

How does Rop regulate the production of H$_2$O$_2$? Rop could be a regulatory subunit of the NADPH oxidase like the mammalian Rac2 and/or could participate in a signaling pathway leading to the activation of NADPH oxidase. Expression of constitutively active mutants of human Rac1 in soybean cells enhanced H$_2$O$_2$ production induced by several different stress stimuli; however, unlike CA-Osracl, this effect is not constitutive and thus is dependent upon the stimuli. These results imply that plants have a regulatory system analogous to the mammalian Rac-dependent regulation of NADPH oxidase. p91phox homologues are indeed present in plants, but plants apparently do not contain p67phox and p47phox homologues (Bolwell, 1999). It is possible that Rop could directly regulate NADPH oxidase in a p67phox-independent manner. Nonetheless, constitutive activation of H$_2$O$_2$ production by CAOsracl suggests that Rop is involved in an additional or alternative signaling mechanism to regulate H$_2$O$_2$ production, likely in an early step of defense signaling pathways; for example, Rop could be regulated by receptor-like kinase R gene product or could regulate Ca$^{2+}$ influx (also an early step of defense signaling) as in pollen tubes.

**Negative regulation of ABA Responses by ROP signaling**

One of the most exciting findings about ROP signaling is the demonstration of its involvement in the negative regulation of ABA responses. A report implicating ROPs in ABA responses describes the respective enhancement and
reduction of ABA-inhibited seed germination by DN-rop2 and CArop2 expression in Arabidopsis (Li et al., 2001). However, this observation did not demonstrate whether ROP is a direct negative regulator of an ABA pathway or a positive regulator of a pathway that antagonizes ABA responses. A role for ROPs in the negative regulation of ABA responses was more convincingly shown in guard cells by Lemichez et al. (2001). Expression of CA-rop6 in Arabidopsis inhibited ABA induced stomatal closure in wild-type plants, whereas DNrop6 expression caused stomatal closure in both the wild type and the abi-1 mutant in the absence of exogenous ABA. This study provided evidence that ABA inactivates one or more ROPs, which apparently act downstream of the ABI1 protein phosphatase, leading to stomatal closure probably through the disruption of actin organization in guard cells (Lemichez et al., 2001). It will be interesting to know whether the ABI1-ROP pathway is guard cell-specific or is also involved in other ABA responses, including those affected by DN-rop2 and CA-rop2 expression.

Studies of loss-of-function mutants have demonstrated ROP9 and ROP10 as more general negative regulators of ABA responses. A rop10 knockout (rop10-1) or a rop9 (RNAi) mutant each showed weak enhancement of ABA inhibition of seed germination, seedling greening, and root growth, although rop10-1/rop9 (RNAi) double mutations strongly enhanced ABA responses in these aspects. Because both ROP9 and ROP10 contain a putative C-terminal farnesylation motif, one or both of these ROPs could be target of ERA1, the _ subunit of protein farnesyltransferase, known to be involved in the negative regulation of ABA responses in both guard cell movement and seed dormancy (Cutler et al., 1996; Pei et al., 1998).

These studies raise an intriguing question: Does the regulation of ABA responses involve multiple ROP-dependent pathways, each controlled by a distinct ROP or a subset of ROPs? The altered ABA responses caused by
expression of *rop2* or *rop6* dominant mutants (Lemichez *et al.*, 2001; Li *et al.*, 2001) could be explained by their interference with the function of ROP9 and/or ROP10. Alternatively, ROP2, ROP6 and ROP9, and ROP10 could respectively regulate distinct ABA pathways. Nonetheless, analysis of various single and multiple *rop* knockout mutants should help to establish the precise role of ROP GTPases in ABA signaling.

**Rop may act as a common molecular switch in many signaling pathways**

Apart from the functions described above, Rop is also implicated in other signaling pathways in plants as well. First, *in situ* immunolocalization of Rop in *Arabidopsis* with anti-Rop1pS antibodies, which react with all *Arabidopsis* Rop isoforms, shows that Rop accumulates in all cell types. However, members of the Rop gene family display various spatial expression patterns (Winge *et al.*, 1997; Li *et al.*, 1998). Second, transgenic plants expressing CA-rop2At or DN-rop2At exhibit pleiotropic phenotypes including alterations in seed germination, leaf morphogenesis, apical dominance, hypocotyls and stem elongation, embryo development, root hair differentiation and development, and responses to phytohormones (such as ABA and brassinolides) and ozone. These phenotypes induced by dominant rop2Atmutants most likely reflect the function of Rop2At and those of Rops that are closely related to Rop2At (e.g., members of Group IV Rop) that are expressed in vegetative tissues. However, Rops that are more distantly related to Rop2At (e.g., members of groups I and II) most likely have distinct functions not revealed by these mutant phenotypes. Third, yeast two-hybrid screens for Rop-interacting proteins have identified several genes encoding proteins related to receptor-like kinases, and Rop-like proteins have been shown to associate with the active CLAVATA1 RLK complex by co-immunoprecipitation using anti-CLV1 and anti-Rop antibodies (Trotochaud *et al.*, 1999). Although which Rop is associated with the CLVATA1 complex remains to be determined, this finding is fascinating because the RLK family, with >100 paralogues in
Arabidopsis, is a major class of transmembrane receptors in plants. Therefore, Rop may achieve its complex signaling through the interaction with RLK or other RLK-associated factors.

Is the mechanism of Rop signaling different from that for the animal RHO family?

Three types of RHO regulators are known in animals: GTPase-activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and guanine nucleotide dissociation inhibitors (GDIs). GEFs activate the GTPase switch by replacing GDP with GTP, whereas GAPs stimulate the intrinsic GTPase activity leading to the deactivation of the GTPase switch. GDIs prevent the activation process by removing GDP bound RHO GTPases from membranes where GEFs are localized. Two RHO GDI homologues are present in the Arabidopsis genome but their functions have not been studied. RHO GAPs have been identified from Arabidopsis and Lotus by the yeast two-hybrid method (Borg et al., 1999). These GAPs preferentially stimulate the Rop subfamily of RHO GTPases and thus are named RopGAPs. All RopGAPs identified to date have a unique feature: the presence of an N-terminal Cdc42/Rac-interacting binding (CRIB) domain localized adjacent to the conserved GAP domain (Borg et al., 1999).

The ability for RopGAPs to be regulated is consistent with the in vivo function of Arabidopsis RopGAP. RopGAP over expression under the pollen specific LAT52 promoter caused pollen tube growth inhibition and a shift of the optimal extracellular Ca2C concentration from 2.0 mM to 0.5 mM. These results suggest that RopGAPs participate in the Ca2C-dependent negative feedback regulation of Rop signaling as a negative regulator. This CRIB dependent GAP regulation and a role for GAP in the negative feedback regulation of GTPase signaling are unique to Rop GTPase signaling in plants.
The activation of Rop signaling may also be distinct from other RHO GTPases. No RHO GEF homologues have been identified in plants, even though about 80% of Arabidopsis sequences have been completed. This raises an interesting possibility that plants have evolved a dramatically different mechanism to activate the GDP-bound Rop GTPases. This notion is consistent with our observation that Rop directly associates with RLKs as described above.

Little is known about Rop effectors, and the current plant databases contain few sequences homologous to typical RHO effectors found in animals and yeast. This suggests that the mode of action for Rop most likely is quite different from that for Cdc42 and Rac. Kost et al. (1999) found that phosphotidylinositol monophosphate kinase (PIP-K) can associate with the recombinant Rop5At/At-Rac2, indicating that it is a putative Rop effector. Evidence supporting a role for PIPK in the control of pollen tube tip growth comes from two observations. First, phosphatidylinositol 4,5-bisphosphate (PIP2), the product of PIP-K, is localized to the tip of pollen tubes where Rop is presumably activated. Second, pollen tube growth is inhibited when the binding site of At-Rac2 for PIP-K is occupied by the fusion protein of GFP-pleckstrin homology domain. Although an in vivo interaction between Rop and PIP-K has yet to be determined, phospholipid kinases are known to serve as RHO effectors in animal systems. However, the potential regulation of PIP-K by Rop is unlike the action of the animal Rop relatives Rac/Cdc42, because Rac/Cdc42 activate PI3K, not PIPK; instead, PIPK is the target of Rho, a more distant relative of Rop (Aspenstrom, 1999). In both yeast and animal systems, each RHO GTPase regulates multiple targets to generate distinct effects (Aspenstrom, 1999). Given the multiplicity of Rop-dependent signaling pathways.
Future prospects and challenges

Although the study of small GTPases in plants is at its early stage, over the last few years rapid progress has been made in our understanding of intracellular signaling pathways mediated by these GTPases, especially by the plant-specific ROP subfamily of Rho GTPases. The surprising finding of Rab11 regulation of brassinolide biosynthesis (Kang et al., 2001) taught us that we cannot assume that the highly conserved Rab, Arf, and Ran GTPases simply control fundamental cellular processes as in yeast and mammals. However, the large number of these GTPases and their potential function in these fundamental processes seemingly present a daunting challenge for investigating their specific roles in signaling. With functional genomic tools available in Arabidopsis, however, it should be possible systematically to analyze their functions using knockout mutants in conjunction with analysis of their subcellular and cellular localization patterns and identification of their functional partners.

ROP, as a sole class of signaling small GTPases in plants, has emerged as an important molecular switch in plant cellular signaling. But many outstanding questions about ROP signaling remain. What the precise roles of individual ROPs need to be addressed using loss-of-function rop mutants. To date, only Arabidopsis ROP9/ROP10 have been functionally analyzed using this approach. A systematic functional analysis using this approach may also lead to the identification of many new ROP-dependent pathways. But this approach alone will be insufficient to elucidate complex functions for individual ROPs, for example, overlapping, and multiple functions. A comprehensive study involving biochemical, proteomic, cell biological, and genetic methods in a model system (for example, Arabidopsis) is needed to determine what signals regulate each ROP, how each ROP is localized at cellular and sub cellular levels, and how each ROP differentially interacts with different regulators and effectors.
The yeast two-hybrid method has revealed several unique ROP regulators and potential targets. However, what factor recruits ROPs to cellular membranes and what is the molecular mechanism underlying ROP activation remain mysterious. Although a ROP-like protein is known to associate with the CLV1 RLK complex, it is unclear what the functional significance of this association is and whether ROP is associated with other RLK complexes. How ROPs control downstream events to modulate specific processes also requires further exploration. RICs promise to be ROP targets that link ROPs to various downstream pathways, but their functions have yet to be determined using loss-of function mutants. Because RICs show no homology to functionally known proteins, it will be critical to identify RIC-interacting proteins to understand how ROPs control each downstream pathway. Each ROP-dependent pathway is presumably controlled by a distinct ROP signaling complex. Development of strategies to isolate specific ROP signaling complexes combined with biochemical, cell biological, and genetic analyses of these complexes should ultimately reveal the mechanisms for ROP recruitment and activation as well as those underlying the functional diversity and specificity of ROP GTPases in plants.
Materials and Methods