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Review

Proteomics of calcium-signaling components in plants

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Abstract

Calcium functions as a versatile messenger in mediating responses to hormones, biotic/abiotic stress signals and a variety of developmental cues in plants. The Ca^{2+}-signaling circuit consists of three major "nodes" – generation of a Ca^{2+}-signature in response to a signal, recognition of the signature by Ca^{2+} sensors and transduction of the signature message to targets that participate in producing signal-specific responses. Molecular genetic and protein–protein interaction approaches together with bioinformatic analysis of the *Arabidopsis* genome have resulted in identification of a large number of proteins at each "node" – ~80 at Ca^{2+} signature, ~400 sensors and ~200 targets – that form a myriad of Ca^{2+} signaling networks in a "mix and match" fashion. In parallel, biochemical, cell biological, genetic and transgenic approaches have unraveled functions and regulatory mechanisms of a few of these components. The emerging paradigm from these studies is that plants have many unique Ca^{2+} signaling proteins. The presence of a large number of proteins, including several families, at each "node" and potential interaction of several targets by a sensor or vice versa are likely to generate highly complex networks that regulate Ca^{2+}-mediated processes. Therefore, there is a great demand for high-throughput technologies for identification of signaling networks in the "Ca^{2+}-signaling-grid" and their roles in cellular processes. Here we discuss the current status of Ca^{2+} signaling components, their known functions and potential of emerging high-throughput genomic and proteomic technologies in unraveling complex Ca^{2+} circuitry.

Abbreviations: ABA, abscisic acid; ABI, ABA-insensitive; ACA, autoinhibited-Ca^{2+}-ATPase; ACBP60, *Arabidopsis* CaM-binding protein60; AGL, agamous-like protein; At, *Arabidopsis thaliana*; AVP1, *Arabidopsis* vacuolar H^+ translocating inorganic pyrophosphatase 1; BON1, bonzai 1; BTB, broad-complex, tramtrack and bric a brac; C2 domain, protein kinase C conserved region 2; CaM, calmodulin; CaMRLK, CaM-binding receptor-like kinase; CAS, Ca^{2+}-sensor receptor; CAT, chloramphenicol acetyl transferase; CAX, Ca^{2+}/H^+ exchanger; CBD, CaM-binding domain; CBF, CRT/DRE-binding factor; CB-HSP, CaM-binding heat-shock protein; CBL, calcineurin B-like; CBP, CaM-binding protein; C/CaMK, Ca^{2+}/CaM-dependent kinase; CG1, CG1-nucleotide-binding domain; ChiP, chromatin-immuno precipitation; CNGC, cyclic nucleotide-gated channel; COR, cold-responsive gene; CP 1, Ca^{2+}-binding protein 1; CPK, Ca^{2+}-dependent protein kinase; CPN1, copine 1; CRK, CPK-related kinase; DAC, depolarization-activated cation channel; DEK1, defective kernel 1; DsPTP, dual specificity protein tyrosine phosphatase; ECA, ER-type Ca^{2+}-ATPase; EF1, elongation factor 1; EF-hand motif, name of the Ca^{2+} binding domain identified as E and F α-helices of the first crystallized Ca^{2+} binding protein parvalbumin; EICBP/SR/CAMTA, ethylene-induced CaM-binding protein/stress responsive/CaM-binding transcriptional activator; EMS, ethyl methanesulfonate mutagenesis; EMSA, electromobility shift assay; ESI, electrospray ionization; FIM1, fimbrin 1; GA, gibberellic acid; GABA, gama-amino butyric acid; GAD, glutamic acid decarboxylase; GFP, green fluorescent protein; GLR, glutamate receptor; GLY, glyoxalase; GST, glutathione-S-transferase; GUS, glucuronidase; HAC, hyperpolarization-activated cation channel; HR, hypersensitive response; HT, highthroughput technologies; IP3R, inositiol trisphosphatereceptor; IPT/TIG, Ig-like, plexins, transcription factor domain; KAT1, K^+-channel of *Arabidopsis thaliana* 1; KCBP, kinesin-like CaM-binding protein; KCO 1, K^+-channel outwardly rectifying channel; KIC, KCBP-interacting Ca^{2+}-binding protein; LUC, luciferase; LRR, leucine-rich repeats; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight; MAS, mannopine synthase gene promoter; MDR-like, multidrug resistant-like protein; MKP1, mitogen-activated protein kinase phosphatase 1; MRP5, MDR-related protein 5; NAM, no-apical meristem domain; NLS, nuclear localization signal; NOS, niticoxide synthase; NPG, no-pollen germination; PA, phosphatidic acid; PCP, pistil expressed Ca^{2+}-binding protein; PKC, protein kinase C; PLA, phospholipase A; PLC, poshpoinositol- phospholipase C; PLD, phospholipase D; PP7, protein phosphatase 7; PPF1, pea, post-floret-specific gene 1; PPI, peptidyl prolyl isomerase; Rboh, respiratory burst oxidase homology protein; Rgs-CaM, regulator of gene silencing-CaM; RING, really interesting gene; RyR, ryanodine receptor; SAUR, small auxin up-RNA; SELEX, systematic evolution of ligands by exponential enrichment; SIPK, SOS-interacting protein kinase; SOS, salt-overlay-sensitive; SRK, S-locus receptor kinase; SUB, short under blue-light; SUL, SUB-like; Syt, synaptotagmins; TAP, tandom affinity purification; TEV, tobacco etch virus protease cleavage site; TGA, TGA nucleotide-binding protein; TPC 1, two-pore channel 1; TPR, tetratrichopeptide repeats; ZAC, zink finger and calcium-binding protein.

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Contents

1. Calcium is a versatile messenger in signaling

Plant cells are equipped with highly efficient mechanisms to perceive, transduce 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) (Bowler and Fluhr, 2000; Reddy, 2001; Rudd and Franklin-Tong, 2001; Sanders et al., 2002; Snedden 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 assignment of these elements in a stimulus-specific signal transduction pathway is a challenging area for plant biologists (Bowler and Fluhr, 2000; Romeis et al., 2001; Zhu, 2003).

Recent studies revealed that various non-proteineous molecules serve as messengers in conveying signals to the cellular machinery in plants (Reddy, 2001). The messengers include Ca^{2+} ions, small organic molecules such as cyclic nucleotide monophosphates, inositol triphosphates and inorganic molecules such as hydrogen peroxide and nitric oxide (Demidchik et al., 2002b; Guo et al., 2003; Levine et al., 1994; Sanders et al., 2002; Scrase-Field and Knight, 2003; Talke et al., 2003; Trewavas and Malho, 1997). With the completion of genome sequences of various organisms including *Arabidopsis*, it has become evident that plants have a large number of EF-hand motif-containing proteins that are likely to bind Ca^{2+} (Day et al., 2002). Besides, using Ca^{2+} imaging techniques, it has been demonstrated that a wide variety of stimuli rapidly alter cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) levels in plant cells (Table 1). Biosynthesis and/or changes in levels of organic messengers and their sensor proteins are poorly understood in

Table 1
Signal-induced Ca^{2+}-mediated cellular processes in plants

Signal	Response[b]	Reference
Developmental		
During cell expansion and elongation	Pollen tube elongation (Ca^{2+} hike in tip) and self-incompatibility (Ca^{2+} hike in shank) (1,2)	Hepler (1997), Messerli et al. (2000), Snowman et al. (2000)
	Root hair and lateral root development (1)	Bibikova et al. (1997), Wymer et al. (1997)
Circadian rhythms	Developmental processes (2)	Johnson et al. (1995), Wood et al. (2001)
Nod factors	Nodulation (1)	Ehrhardt et al. (1996), Shaw and Long (2003)
Fertilization	Fusion of the sperm cell with egg (1)	Digonnet et al. (1997)
Gravity	Gravitotropism (1)	Gehring et al. (1990), Plieth and Trewavas (2002)
Hormone		
Auxin	Cell-elongation and cell division	Felle (1988)
Abscisic acid[a]	Stomatal regulation (1,2,3)	Allen et al. (1999), Klusener et al. (2002), McAinsh et al. (1990, 1992), Wang et al. (1991)
Gibberellic acid	Cell division, α-amylase secretion in germinating seeds (2)	Bush and Jones (1988), Gilroy and Jones (1992)
Abiotic stress		
NaCl	Gene expression, osmolyte synthesis, ion transport, K^+ uptake, stomatal regulation, salt tolerance (1, 2, 3)	Knight et al. (1997), Liu and Zhu (1998), Lynch et al. (1989)
Cold	COR gene expression, proline synthesis, changes in membrane lipid profile and cold acclimation (1,2)	Campbell et al. (1996), Knight et al. (1999), Knight et al. (1991), Thomashow (1998)
Anoxia	Anaerobic adaptation (1)	Subbaiah et al. (1994, 1998)
Drought and hypoosmotic	Gene expression, synthesis of osmoprotectants, stomatal regulation and osmotolerance (1,2)	Knight et al. (1997), Takahashi et al. (1997)
Aluminium[a]	Ion balance (1,2)	Kawano et al. (2003)
Oxidative stress	Production of ROSs, HR (1,2)	Cessana and Low (2001), Levine et al. (1994), McAinsh et al. (1996), Pei et al. (2000), Price et al. (1994)
Ozone stress	Production of ROSs (2)	Clayton et al. (1999)
Heat shock	Thermotolerance (1,2)	Gong et al. (1998), Larkindale and Knight (2002)
Wind/touch	Thigmomorphogenesis (1,2)	Knight et al. (1991), Knight et al. (1992), Legue et al. (1997), Polisensky and Braam (1996), Russell et al. (1996)
Light stress		
Red light	Photomorphogenesis (1)	Shacklock et al. (1992)
Blue light	Photomorphogenesis (2)	Harada et al. (2003), Stoelzle et al. (2003)
UV-B light	Flavonoid synthesis (2)	Frohnmeyer et al. (1999)
Biotic stress		
Pathogens and elicitors	*AvrRpm1-RPM1* mediated disease response, production of ROS, phytoalexin synthesis, production hypersensitive response (1,2)	Blume et al. (2000), Grant et al. (2000), Knight et al. (1991), Lamb and Dixon (1997), Lecourieux et al. (2002), Levine et al. (1996), Mithofer et al. (1999), Zimmermann et al. (1997)

[a] ABA signal and aluminum stress signal show elevation and depletion of cytosolic Ca^{2+} levels.
[b] The numbers in parenthesis indicate the method used to measure cytosolic free Ca^{2+} levels: fluorescent indicator dyes (1); transgenic plants expressing aequorin (2) or cameleon (3). COR, cold regulated genes; ROS, reactive oxygen species; HR, hypersensitive response.

plants. The fact that almost all signals induce changes in $[Ca^{2+}]_{cyt}$ in plants, suggesting that Ca^{2+} is widely used in signal transduction mechanisms as compared to other messengers (Knight, 2000; Reddy and Reddy, 2002). Therefore, great attention has been given to deciphering the role of Ca^{2+} and its regulation of downstream components in Ca^{2+}-mediated signal transduction cascades that couple signal to cellular processes in plants.

Calcium has been implicated in mediating various developmental processes (pollen tube growth, root-hair and lateral root development and nodulation), hormone-regulated cellular activities (cell division and elongation, stomatal closure/opening), pathogen- and elicitor-induced defense related processes (production of reactive oxygen species, phytoalexin synthesis and hypersensitive response) and a variety of abiotic stress signal-induced

gene expression (Table 1). Calcium imaging techniques using transgenic plants expressing cameleon or aequorin and injection of fluorescent indicator dyes coupled with mutants that are defective in Ca^{2+} mediated cellular processes greatly helped our understanding of signal-induced Ca^{2+} signature specificity (Table 1). However, the identity and functions of downstream transducers and mechanisms by which Ca^{2+} mediates a variety of cellular responses are just beginning to be unraveled in plants. This review focuses on genome-wide analysis of Ca^{2+} signaling components such as channels, pumps, transporters, sensors and targets with special emphasis on *Arabidopsis*. An overview of the current understanding of Ca^{2+} signaling, missing areas and future direction for functional analysis of Ca^{2+} signaling components using state-of-the-art genome-wide high-throughput technologies is also presented.

2. The central dogma of Ca^{2+} signaling

The central dogma of Ca^{2+}-signaling consists of three major "nodes" (generation of Ca^{2+} changes, recognition of these changes and transduction) that produce a specific cellular response to a Ca^{2+} message. Any given Ca^{2+}-mediated cellular process begins with the generation of a signal-specific "Ca^{2+}-signature" in the cytoplasm (or nucleus) by the synchronized activity of channels, pumps and transporters (Miedema et al., 2001; Reddy, 2001; Sanders et al., 2002; Scrase-Field and Knight, 2003) (Fig. 1; Tables 1 and 2). The signature acts as a cellular "chemical switch" (Scrase-Field and Knight, 2003). Changes in $[Ca^{2+}]_{cyt}$ levels are sensed by a specific set of protein(s) with or without EF-hand motifs termed as "Ca^{2+}-sensors" (Day et al., 2002). Upon activation by Ca^{2+}-binding, Ca^{2+}-sensors regulate activity/function of specific protein "targets" in the cell that are involved in producing a signal-specific cellular/physiological/developmental response (Luan et al., 2002; Reddy et al., 2002).

2.1. Generation of Ca^{2+} signature: role of Ca^{2+}-permeable channels, Ca^{2+}-ATPases and antiporters

The Ca^{2+} concentration in resting cells is in the nanomolar range (100–200 nM) whereas it is in the millimolar range (1–10 mM) in extracellular and intracellular Ca^{2+} stores (Reddy, 2001; Rudd and Franklin-Tong, 2001; Trewavas and Malho, 1997). The $[Ca^{2+}]_{cyt}$ levels are highly dynamic and are regulated in response to various signals by channels, pumps and transporters at the "first node" (Fig. 1) (Miedema et al., 2001). The $[Ca^{2+}]_{cyt}$ levels are elevated up to 3 μM and the magnitude and duration of Ca^{2+} changes vary depending on the signal or cell type. Various types of Ca^{2+}-permeable channels elevate $[Ca^{2+}]_{cyt}$ levels in response to various

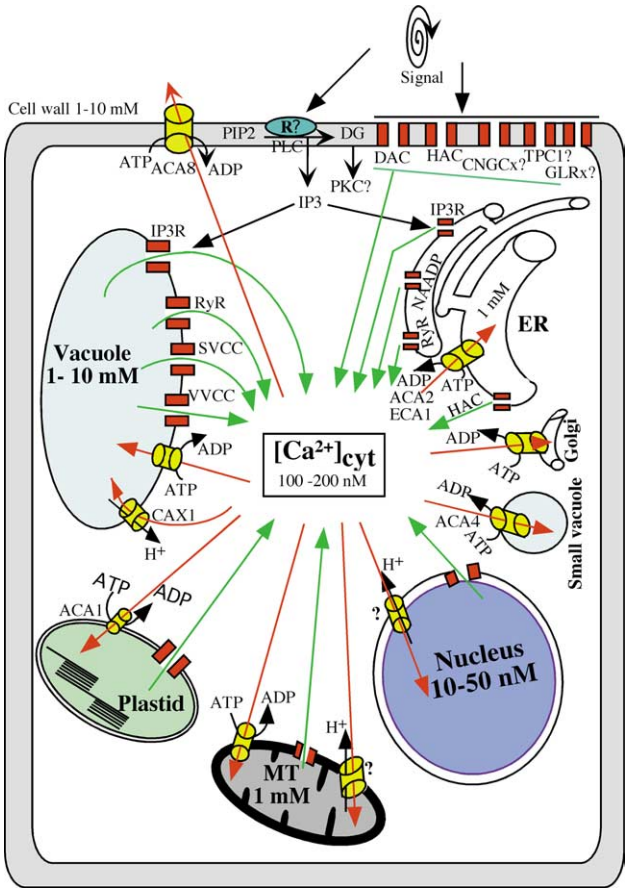

Fig. 1. Schematic representation of Ca^{2+}-permeable channels, pumps and transporters on various membranes of a cell that are thought to be involved in generation of a signal-specific Ca^{2+} signature and restoring it to resting level. Different types of Ca^{2+}-permeable channels are shown with red-gates that allow Ca^{2+} entry from high-concentration sources (cell walls and organelles) into low concentration cytosol. The channels are gated by voltage (HAC, DAC) and ligands such as Ca^{2+} (TPC1) Ca^{2+}-CaM, cNMP (CNGCs), and glutamate (GLRs), IP3 (IP3R), cADPR (RyR) and NAADP (NAADP receptor). Maintenance of low cytosolic Ca^{2+} is accomplished by Ca^{2+}-ATPases and transporters (shown with yellow-cylinders), which are driven by energy from the hydrolysis of ATP and proton-force, respectively. The precise location of some channels, ATPases and antiporters, the organellar Ca^{2+} sources and the molecular identity of IP3R, RyR and NAADP receptor remain to be identified (?). The estimated Ca^{2+} concentration in the cytosol, different organelles and the cell wall is indicated (Sanders et al. (2002); Scrase-Field and Knight (2003); Trewavas and Malho (1998); White (2000); White and Broadley (2003)). Green and red arrows indicate influx and efflux sequestration of Ca^{2+} stream, respectively. Refer to Table 2 for the full names of channels. PLC, phospholipase C; ER, endoplasmic reticulum; MT, mitochondrion.

signals (Sanders et al., 2002; Scrase-Field and Knight, 2003; Trewavas and Malho, 1997) (Table 2). Voltage- and ligand-gated Ca^{2+}-permeable channels on the vacuolar membrane (inositol triphosphate receptor and ryanodine sensitive receptor), on the plasma membrane (depolarization- and hyperpolarization-activated channels and yet unknown CNGCs and GLRs) and on the ER (NAADP receptor, inositol triphosphate receptor and ryanodine sensitive receptor) contribute to elevation

Table 2
Proteins involved in generating signal-induced Ca^{2+} signatures

Protein	Domains	Location	Function	Regulation	Reference
Calcium-permeable channels					
Two-pore channel 1 (TPC1)	TM, EF-hand motifs	PM?	Ca^{2+} influx	Sugar-induced depolarization and Ca^{2+}	Furuichi et al. (2001)
Hyper-polarization activated cation channel (HAC)	–	PM, VA, ER	Guard cell opening, Ca^{2+} gradient in tip-based systems	ABA and H_2O_2 in guard cells, $[Ca^{2+}]_c$ in root hairs and voltage	Gelli and Blumwald (1997), Hamilton et al. (2000), Kiegle et al. (2000), Very and Davies (2000)
Depolarization activated cation channel (DAC)	–	PM, VA	Ca^{2+} influx	Voltage	Thion et al. (1998)
Inositiol triphosphate receptor (IP3R)	–	VA, ER	Ca^{2+} influx by salt and osmotic stress signals and gravitropism	IP3	Drobak and Watkins (2000), Perera et al. (1999), Sanders et al. (1999)
Ryanodine-sensitive receptor (RyR)	–	VA, ER	Ca^{2+} influx and mediates defense gene regulation and ABA signal transduction	cADPR	Navazio et al. (2001), Sanders et al. (2002)
NAADP receptor	–	ER	Ca^{2+} influx	NAADP	Navazio et al. (2000)
Cyclic nucleotide gated channels (CNGCs; 20 members)	TM, cNMP, CBD	PM?	Ca^{2+} influx, CNGC2 is involved in developmentally induced cell-death	cNMPs, Ca^{2+}-CaM	Demidchik et al. (2002a), Leng et al. (2002), Moutinho et al. (2001)
Glutamate receptors (GLRs; 30 members)	TM, Glu-binding domain	PM?	Transport of Ca^{2+}	Glutamate?	Dennison and Spalding (2000), Kim et al. (2001), Lacombe et al. (2001)
Transporters (Ca^{2+}-ATPases and Ca^{2+}/H^+ antiporters)					
Autoinhibited Ca^{2+}-ATPases (ACA pumps; 12 members)	TM, CBD and ATPase	PM, VA, ER, PL, GO, MT	High affinity and low capacity Ca^{2+} transporters	Energy from hydrolysis of ATP and Ca^{2+}-CaM	Geisler et al. (2000), Sanders et al. (2002)
ER-type Ca^{2+}-ATPases (ECA pumps; 4 members)	TM and ATPase	ER (ECA1) and others?	High affinity and low capacity Ca^{2+} pumps and house keeping activity	Energy from hydrolysis of ATP	Axelsen and Palmgren (2001), Geisler et al. (2000)
Ca^{2+}/H^+ antiporters (12 members)	TM, auto-inhibitor	PM, VA, (MT and NU?)	CAX1 shows low affinity, high capacity Ca^{2+} transportation and delayed HR	Proton-motive force	Cheng et al. (2003), Hirschi et al. (1996)

Although the genes encoding Ca^{2+} permeable channels HAC and DAC have not been characterized, it is speculated that annexins and TPC1 encode HAC and DAC, respectively. The molecular identity of IP3 and ryanodine-sensitive receptors is not known. TM. transmembrane domain; CBD, calmodulin-binding domain; PM, plasma membrane; VA, vacuole; ER, endoplasmic reticulum; MT, mitochondria; NU, nucleus; GO, golgi apparatus; PL, plastids.

of $[Ca^{2+}]_{cyt}$ levels (Fig. 1; Table 2). It is thought that channels maintain spatial temporal increase in $[Ca^{2+}]_{cyt}$ as well as duration and frequency of elevated $[Ca^{2+}]_{cyt}$. Although the precise location of non-selective cationic cyclic nucleotide gated channels (AtCNGC, 20 members) glutamate receptors (AtGLR, and 30 members) and their role in elevation of $[Ca^{2+}]_{cyt}$ are poorly understood, they might provide a signal cross-talk between Ca^{2+}, cNMP and/or glutamate messengers (Demidchik et al., 2002a; Kim et al., 2001; Lacombe et al., 2001; Leng et al., 2002; Moutinho et al., 2001). Much research is needed to unravel the channel and receptor specificities in regulating $[Ca^{2+}]_{cyt}$ levels in various cell types in response to developmental cues, hormones and stress signals in plants.

The proton-gradient force-driven H^+/Ca^{2+} antiporters and ATP-driven Ca^{2+}-ATPases act in opposite way to Ca^{2+}-permeable channels by pumping elevated $[Ca^{2+}]_{cyt}$ into the exterior and/or intracellular organelles (Fig. 1). The activities of pumps and transporters terminate Ca^{2+} signature as well as replenish Ca^{2+} stores for generation of future Ca^{2+} signatures (Reddy, 2001). Although, the location and activity of 11 of the 12 members of H^+/Ca^{2+} antiporters (CAXs) in *Arabidopsis* are not known, CAX1 localizes to the vacuolar membrane and overexpression results in plants with Ca^{2+}

disorder symptoms such as reduced growth and sensitivity to cold stress due to hyperantiport activity (Hirschi, 1999; Hirschi et al., 2001; Shigaki et al., 2002). There are two types of Ca^{2+}-ATPase pumps. The autoinhibited Ca^{2+}-ATPases (ACA) type pumps are distinguished from ER-type Ca^{2+}-ATPase (ECA) pumps in that they are regulated by an autoinhibitory domain and a Ca^{2+}-CaM-binding domain at the N-terminus and are located on both plasma membrane and endomembranes. The ECAs are sensitive to cyclopiazonic acid and thapsigarin whereas ACAs are insensitive. Twelve ACA and four ECA pumps are present in *Arabidopsis* and locations of some of these members have been identified (Fig. 1) (Sanders et al., 2002).

The presence of 51 potential channels and 30 pumps and transporters in *Arabidopsis* (Table 2) suggests complexity in the regulation of signal-specific Ca^{2+}-signature. At the same time, only a few of the components at this "first node" have been characterized. Research to define specific as well as redundant functions of these membranous proteins in Ca^{2+} mediated signaling is an area of intense investigation. Localization of voltage-gated channels, Ca^{2+}-ATPases and antiporters on various membranes that produce a specific Ca^{2+}-signature in response to an array of signals are yet to be studied.

2.2. Sensors of the Ca^{2+} signature

The signal-specific Ca^{2+} signature is readily decoded by an array of Ca^{2+}-binding proteins or Ca^{2+} sensors at the "second node". In plants, two classes of sensors that differ in their Ca^{2+}-induced activation are present. The first class of sensors (e.g., calmodulin and calcineurin B-like proteins without any responder domains) bind Ca^{2+} and undergo conformational change and in turn regulate the activity/function of a variety of other target proteins or regulate gene expression. Therefore, these sensors are called "sensor relays". The second group of sensors is called "responders" with other effector domains (e.g., protein kinase or phospholipase domain) through which they relay the message to their downstream targets. Therefore, the decoding of Ca^{2+} signals begins with intermolecular and/or intramolecular mode of interaction mechanisms that lead to an array of pathways through which Ca^{2+} regulates a number of responses. However, the identity of sensors in many Ca^{2+}-mediated cellular processes in plants is not known. In recent years, identification of sensors has been the primary area of research in Ca^{2+} signaling.

2.2.1. Ca^{2+} sensors and their structural features

The Ca^{2+} binding EF-hand motif in most of the Ca^{2+} sensors is highly conserved. The EF-hand motif is a 29 aa helix-loop-helix structure. The loop in each EF-hand motif consists of 12 residues with a pattern of similar residues at positions $X^*Y^*Z^* - Y^* - X^{**} - Z$ that coordinate Ca^{2+} binding. Because the EF-hand motif is highly conserved in Ca^{2+} binding proteins, it was logical to search the *Arabidopsis* genome database for proteins with EF-hand motifs. Our bioinformatics analysis using domain prediction programs and BLAST searches with known Ca^{2+}-binding proteins containing EF-hand motifs (CaM4, CBL3, CPK1 and KCBP interacting Ca^{2+}-binding protein) against the *Arabidopsis* database coupled with a literature search has revealed a total of 250 proteins with varying numbers of EF-hand motifs (Day et al., 2002). As compared to other completely sequenced genomes, *Arabidopsis* has the largest number of EF-hand containing proteins. Although the presence of pairs of EF-hand motifs in proteins increases stability as well as affinity for Ca^{2+} (Nakayama et al., 2000), a majority of the *Arabidopsis* EF-hand proteins contain an odd number (1, 3 or 5) of motifs. The major subfamilies of EF-hand Ca^{2+} sensors that bind Ca^{2+} are CaMs, CaM-like proteins, CBLs and CPKs (Table 3).

Two other motifs that mediate Ca^{2+}-dependent interactions of proteins with membranes or membrane lipids have been identified in plants. A 70-amino acid annexin fold (Clark and Roux, 1995; Huber et al., 1990) is present in members of the membrane associated annexin subfamily of Ca^{2+} sensors. A C2 domain (similar to conserved region 2 in protein kinase C) of about 130–145 aa, consisting of subdomain A (DPYVK), B (polybasic core, KXK(R)T) and C (LNPXWN(X)EXFXF), is present in membrane associated proteins. Unlike the EF-hand helix-loop-helix structure, the C2 domain forms a β-sheet scaffold with eight antiparallel strands connected by loops. Loops 1–3 are placed on top of the β-sheets and are shown to coordinate Ca^{2+} binding (Sutton et al., 1995). Although EF-hand and C2 motifs bind Ca^{2+} and are present as single or multiple motifs in proteins, they differ in several aspects. The EF-hand modules bind Ca^{2+} ions in the loops and undergo a large conformational change that results in exposure of hydrophobic pockets, which facilitates its interaction with a variety of proteins. In contrast, the loop regions of the C2 structure bind 3–4 Ca^{2+} ions but do not undergo conformational change upon Ca^{2+} binding. The binding of Ca^{2+} to the C2 domain in a protein allows its interaction preferentially with negatively charged phospholipids. As the C2 domain also binds lipids, it is also called CalB (calcium and lipid binding).

There are 142 C2-domain containing proteins in the *Arabidopsis* genome (SMART accession number for C2 domain: SM00239; http://smart.embl-heidelberg.de). *Arabidopsis* C2 motif proteins include some members of phospholipases Cs and Ds, synaptotagmins, calpains, copines, PIP2-poshpodiesterase, PI3 kinase and several other unknown proteins (Craxton, 2001; Lid et al., 2002; Meijer and Munnik, 2003; Tomsig and Creutz, 2002)

Table 3
Known and putative Ca^{2+} sensors in *Arabidopsis* (a complete list of proteins is available at:www.arabidopsis.org/info/genefamily/ef-hand.html; Day et al. (2002) and www.arabidopsis.org/info/genefamily/genefamily.html)

Group of Ca^{2+} sensor[a]	M[b]	Function/activity (reference)
Group I		
Proteins with myb-domain	2	ND[d]
Proteins with NLS	4	ND
Elongation factor EF2	1	ND
Protein with F-box domain	1	ND
Proteins with ATP or GTP binding sites	1	ND
Proteins with EPS15 repeat	3	ND
Others	11	ND
Group II		
KCO 1	3	K^+ transport (Czempinski et al., 1997)
AtPLC 1	1	IP3 synthesis, expression is induced by salt and dehydration (Hirayama et al., 1995; Otterhag et al., 2001)
AtFIM 1	2	ND (McCurdy and Kim, 1998)
Protein with NAM domain	1	ND
Protein with bHLH domain	1	ND
Proteins with Zinc finger domain	2	ND
Plant peroxidase	1	ND
eEF4	1	ND
Mitochondrial carrier proteins	4	ND
Nucleoside transporters	3	ND
Eukaryotic protein kinase	1	ND
NLS containing protein	1	ND
Proteins with PPR domain	2	ND
Others	25	ND
Group III		
Calcineurin B-like proteins (CBLs)	10	Ca^{2+} sensor relays and involved in salt, cold and drought tolerance (Kudla et al., 1999; Liu and Zhu, 1998)
NaCl-inducible protein (AtCP1)	1	Gene expression is induced by salt treatment (Jang et al., 1998)
Protein phosphatase 2As	5	Dephosphorylation (Hendershot et al., 1999)
KCBP interacting Ca^{2+}-binding protein (KIC)	1	Involved in the regulation of trichome branching (Reddy et al., 2004)
Protein with NLS	1	ND
Others	22	ND
Group IV		
Calmodulins (CaMs)[c]	7	Ca^{2+} sensor relays (Jena et al., 1989; Zielinski, 1998 Zielinski, 2002a)
CaM-like proteins[c]	9	Members of this family are inducible by mechanical stress (Braam and Davis, 1990; Day et al., 2002)
Centrins	2	Not known (Cordeiro et al., 1998)
Arabidopsis pollen Ca^{2+} binding protein (APC1)	1	Functions in pollen (Rozwadowski et al., 1999)
Protein homolog to tobacco rgs-CaM	1	Tobacco gene is involved in mediating viral-induced gene silencing (Anandalakshmi et al., 2000)
Plasma membrane associated protein (PM129)	1	ND (Bartling et al., 1993)
Others	34	ND
Group V		
CPKs	34	Ca^{2+} sensor responders/relays (Harper et al., 1991; Hrabak et al., 1996; Sanders et al., 2002)
CRKs	3	ND
Others	3	ND
Group VI		
AtRbohs	6	ROS production (Keller et al., 1998; Torres et al., 1998)
AtRboh-like proteins	3	Not known
Protein phosphatase 2C (ABI 1)	1	Induced by ABA and regulates stomatal closure (Leung et al., 1997; Meyer et al., 1994)
Glutamate dehydrogenase	1	Not known (Turano et al., 1997)
GTPases	3	Not known
(Two pore channel 1 (TPC1)	1	Ca^{2+} influx carrier (Furuichi et al., 2001
Protein with BTB domain	1	ND
Others	15	ND

(*continued on next page*)

Table 3 (*continued*)

Group of Ca^{2+} sensor[a]	M[b]	Function/activity (reference)
Annexin folds-containing proteins		
Annexins		Vesicle fusion and secretion (Clark and Roux, 1995; Lim et al., 1998)
Proteins with C2 domain[c]		
PLA2[f]	4	Hydrolyzes phospholipid into free fatty acid (Meijer and Munnik, 2003)
PI-PLCs	7	Hydrolyzes PIP2 to IP3 and DAG (Meijer and Munnik, 2003)
PLDs	10	Hydrolyzes phospholipids into PA (Meijer and Munnik, 2003)
PI3 kinase[f]	2	Phosphorylation of PI to PI3P (Welters et al., 1994)
Synaptotagmins	5	Not known (Craxton, 2001)
Copine	1	Humidity and temperature controlled plant development (Hua et al., 2001; Jambunathan et al., 2001)
Calpain	1	A cysteine proteinase involved in grain aleurone development (Lid et al., 2002; Wang et al., 2003a)
Proteins that bind Ca^{2+} without any known Ca^{2}-binding domain		
Caleosins	6	Associated with lipid bodies and some of them are inducible by ABA and dehydration (Naested et al., 2000; Takahashi et al., 2000)
14-3-3s	8	Ca^{2+} stimulates their interaction with phosphorylated nitrate reductase (Athwal and Huber, 2002; Lu et al., 1994)
Short under blue light 1 (SUB1)	3	Blue light dependent photomorphogensis (Guo et al., 2001a)
Calreticulin		Ca^{2+} homeostasis (Baluska et al., 1999)
Pistil expressed Ca^{2+} binding protein (PCP)		Pollen-pistil interactions (Furuyama and Dzelzkalns, 1999)
Calcium sensor receptor (CAS)		Guard cell signaling (Han et al., 2003)

[a] The Ca^{2+} sensors are grouped (group I to VI) based on phylogenetic analysis of Arabidopsis full-length EF-hand containing proteins; Day et al. (2002).

[b] Subfamily members (M) may vary in the number of Ca^{2+}-binding motifs.

[c] The CaM and CaM-like proteins are grouped based on the number of amino acids (149 aa) and 4 EF hand motifs. CaM1-7 have 149 aa and 4 EF hands. CaM-like proteins such as At1g12310, At1g62820, At5g37770 (TCH2), At4g14640 (TCH3), At2g41090 (CaBP22), At3g22930, At4g14640 and At3g51920 have either less or more than 149 aa and 4 EF-hand motifs.

[d] ND, not determined.

[e] There are 142 C2-domain containing proteins in the *Arabidopsis* genome (SMART accession number for C2 domain: SM00239 and http://smart.embl-heidelberg.de.

[f] Conserved C2 domain is not present in these proteins.

(Table 3). Although the function of the C2 domain in several proteins is not known, it is thought that Ca^{2+} neutralizes negatively charged residues in the loop regions of the C2 domain, permits its interaction with phospholipids in the membrane and functions in trafficking (Rizo and Sudhof, 1998).

Although 4 PLA2s with C2 domains are present in *Arabidopsis*, regulation of their catalytic activity (hydrolysis of phospholipids into free-fatty acid and a lysophospholipid) by Ca^{2+} has not been reported (Kopka et al., 1998). PLCs have one C2 domain at the C terminus and their enzyme activity (conversion of PI(4,5)P2 into inositol 1,4,5-triphosphate and diacyl glycerol) has been shown to be activated by nanomolar concentrations of Ca^{2+} (Kopka et al., 1998). IP3 has been shown to release Ca^{2+} from intracellular stores whereas DAG is converted into PA by DAG kinase. PLDs contain an N terminus C2 domain and hydrolyze phospholipids to produce phosphatidic acid (PA), which acts as a messenger in plants (Meijer and Munnik, 2003). Plants contain 10 PLDs with C2 domains and these are grouped into α, β, γ and δ. PLDs with C2 domains are found in plants and mosses but not in fungi and animals suggesting C2 domain-containing PLDs perform plant-specific functions (Meijer and Munnik, 2003).

In *Arabidopsis*, five synaptotagmins (Syt) with two C2 domains have been identified. Syts are present in plants and animals and absent in yeast (Craxton, 2001). Although functions of plant Syt members have not been studied, these proteins are implicated in membrane trafficking. A rice (OsERG1, elicitor-responsive gene 1) protein (159 aa) with a C2 domain has been shown to translocate to the plasma membrane in response to a fungal elicitor derived from *Magnaporthe grisea* or to Ca^{2+} ions (Kim et al., 2003a). A homolog of OsERG1 is also present in *Arabidopsis* (AAG52148), maize (U64437) and pumpkin (Cmpp16-1 and 2). *Arabidopsis* ZAC (zinc and calcium) protein contains a C2 domain and PI3P binding zinc-finger domain and possesses GTPase activating activity on *Arabidopsis* ADP-ribosylation factor proteins, suggesting its role in G-protein-mediated signaling and vesicular transport (Jensen et al., 2000). Although protein kinase C-like enzyme activity has been shown in plants (Subramaniam et al., 1997), its corresponding gene in the *Arabidopsis* genome has not been identified.

Experimental analysis of some *Arabidopsis* proteins showed that they bind Ca^{2+} without the conserved EF-hand motif. These proteins include, calreticulin, pistil-induced Ca^{2+}-binding protein (PCP1), caleosins, 14-3-3 proteins, short under blue light 1 (SUB1) and its related proteins SUL1 and SUL2 (Table 3).

The domain prediction approach coupled with experimental approaches revealed that the Ca^{2+} sensors are involved in several cellular processes including Ca^{2+} signal relays, phosphorylation and dephosphorylation cascades, production of second messengers (IP3, PA and reactive oxygen species), ion transport pathways, translation and transcription, phospholipid-based signaling and membrane trafficking and developmental processes such as pollen-tube growth and trichome morphogenesis (Table 3). As in CPKs, some Ca^{2+} sensors (SUB1 and At1g74430) contain other domains such as DNA-binding domains (Table 3). These proteins might be involved in regulating the expression of genes involved in a Ca^{2+} mediated cellular response (Day et al., 2002; Guo et al., 2001a). The presence of a wide variety of Ca^{2+} sensors also suggests that the Ca^{2+} signaling networks are extensively utilized in plants.

2.2.2. Ca^{2+} sensors show differential expression, localization and Ca^{2+} affinity

Studies on the expression and localization of Ca^{2+} sensors and their affinity to Ca^{2+} are providing insight into their functions in cellular responses. For example, the expression of the conserved *Arabidopsis* CaMs and as well as conserved and divergent CaMs of potato and soybean is different in response to touch stimulus or they differ in their spatial and temporal expression (Snedden and Fromm, 1998; Takezawa et al., 1995; Zielinski, 1998). Further, soybean divergent CaMs (SCaM4 and SCaM5), but not the conserved CaMs (SCaM1-3), are implicated in plant defense response, suggesting that they also differ in their targets (Heo et al., 1999). Similarly, CPKs and CBLs differ in their expression, targets and thereby their role in response to stress signals (Cheng et al., 2002; Luan et al., 2002). Among eight CPKs tested, AtCPK10 and AtCPK11 are involved in mediating drought and salt stress whereas AtCPK30 (AtCDPK1a) is involved in cold, salt and ABA-induced gene expression (Sheen, 1996). NtCPK2 and NtCPK3 are involved in mediating disease resistance and osmotic stress whereas NtCPK1 mediates an array of signals including GA, ABA, cytokinin, wounding, fungal elicitor and salt stress (Ludwig et al., 2004; Romeis et al., 2001). Similar results were also obtained for different tobacco CaMs (Yamakawa et al., 2001). Depending on the presence of a prenylation domain, petunia CaM53 localizes to the plasma membrane or nuclear compartment, suggesting differential localization of CaMs (Rodriguez-Concepcion et al., 1999a, 2000) and regulation of different cellular activities based on the prenylation status. Members of the CPK group localize to the ER, plasma membrane and other membranes (Cheng et al., 2002; Dammann et al., 2003; Hrabak et al., 2003; Lu and Hrabak, 2002).

CBLs have three EF-hand motifs and show no sequence similarity with other Ca^{2+} sensors, except in the EF-hand regions. However, like CPKs, they also contain myristoyl domains for membrane localization (Hrabak et al., 2003; Luan et al., 2002; Zhu, 2003) and are similar to CaMs in not containing any other responder domains. In *Arabidopsis*, 10 CBL isoforms that show differential expression were identified (Luan et al., 2002). The expression of CBL1 is induced by wounding, cold, drought, salt, light and ABA whereas CBL2 is induced by only light (Cheong et al., 2003). The CBL4 (SOS3) is an extensively characterized Ca^{2+} sensor that mediates the salt tolerance SOS pathway through a SOS kinase cascade (Qiu et al., 2002; Zhu, 2002).

Different Ca^{2+} sensors and members of subfamilies of Ca^{2+} sensors may also differ in their affinity for Ca^{2+} due to the variation in number and amino acid sequence of the EF-hand motifs. For example, three soybean CPKs show unique Ca^{2+}-binding properties (Lee et al., 1998). Similarly, *Arabidopsis* CaM2 and KIC (KCBP-interacting Ca^{2+} binding protein) require varied concentrations of Ca^{2+} to inhibit the enzyme activity of their common target, kinesin-like calmodulin-binding protein, KCBP, a microtubule motor protein (Reddy et al., 2004). Differences in localization, tissue expression and Ca^{2+} affinity are more prevalent in C2 domain containing *Arabidopsis* PLD subfamily members. Activity of PLDα is PIP2-independent and requires millimolar concentrations of Ca^{2+} whereas PLDβ, γ and δ are PIP2-dependent and require submicromolar concentrations of Ca^{2+} (Pappan and Wang, 1999; Pappan et al., 1997; Zheng et al., 2000). The differences in Ca^{2+} affinity of C2 domains in PLDs are due to variations in Ca^{2+} coordinating residues in the C2 domain. *Arabidopsis* PLDs also differ in their subcellular localization and tissue expression pattern. Although both PLDα and PLDγ are present in plasma membrane and intracellular membranes such as mitochondria and clathrin-coated vesicles, they differ in their relative amounts. In addition, PLDγ, but not PLDα, is present in the nuclear fraction (Fan et al., 1999). Expression of PLD genes is differentially regulated by various stress factors. PLDβ gene expression is low in all organs tested compared to PLDα and PLDγ (Fan et al., 1999). Gene expression of PLDα, but not other PLDs, is induced by osmotic stress (Sang et al., 2001), whereas PLDδ is induced by drought and salinity (Katagiri et al., 2001) and PLDβ1 is induced by the elicitor xylanase (Wang, 2000). These findings suggest that PLDs are involved in specific lipid-based signaling in response to abiotic and biotic stresses in plants.

2.3. Targets of the Ca^{2+} sensors

Variations in number of Ca^{2+} binding motifs and other domains, Ca^{2+} affinity, expression and localization of Ca^{2+} sensors suggest their possible interaction with different sets of targets at the "third node". Using protein–protein interaction-based approach, yeast two-hybrid assay or in vitro protein kinase assay, some of the targets of CaMs, CBLs and CPKs have been identified.

2.3.1. Ca^{2+}-calmodulin-binding proteins

The Ca^{2+} sensor, CaM, is a highly conserved acidic protein in eukaryotes. However, the CaM-target proteins within plants and/or animals are not conserved (Kim et al., 2002a; Reddy et al., 2002; Snedden and Fromm, 2001; Yang and Poovaiah, 2003). Studies have shown that the mapped CaM-binding domains in CaM-targets (CaM-binding proteins, CBPs) exhibit variation at the primary amino acid sequence level. This is in contrast to the Ca^{2+} sensors where the Ca^{2+}-binding EF-hand domain is highly conserved. Because of the lack of sequence conservation in the CaM-binding domain (CBD), a number of laboratories have used a protein–protein interaction-based screening of expression libraries with CaM. To isolate CaM-binding proteins, several expression libraries prepared from tissues (root, leaf, stem, flower, pollen) at different developmental stages or treated with hormones (auxin, ethylene and GA), elicitor or pathogens have been screened using ^{35}S-labeled CaM in the presence of Ca^{2+} (Fig. 2(a)) (Ford-

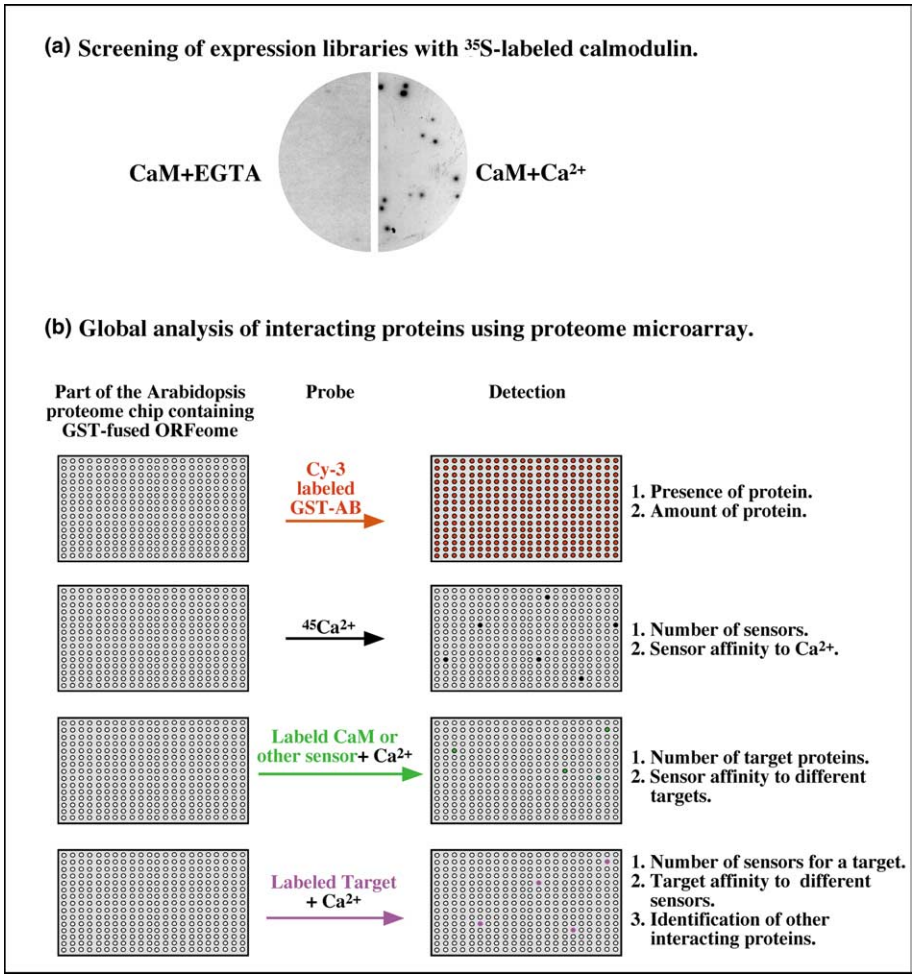

Fig. 2. Identification of Ca^{2+} sensors and targets using protein–protein interaction-based approaches. (a) Representative autoradiogram of screening of expression libraries with ^{35}S-labeled calmodulin (CaM). The proteins encoded by recombinant phages in a library were induced by IPTG, transferred to nitrocellulose membranes and hybridized with ^{35}S-labeled CaM. After purification of a putative clone encoding CaM-binding protein, it was probed with CaM in the presence of EGTA (CaM + EGTA) or Ca^{2+} (CaM + Ca^{2+}) to verify Ca^{2+} dependent interaction between CaM and its target (Fromm and Chua (1992); Reddy et al. (2002); Zielinski (2002b)). (b) Diagrammatic representation of screening of an *Arabidopsis* proteome chip containing GST-fused ORFeome (~30,000 proteins) for Ca^{2+} sensors, targets and their interacting proteins. First panel shows probing of a proteome chip with Cy3-labeled GST-antibody (GST-AB). Second panel depicts screening of a proteome with $^{45}Ca^{2+}$ to identify Ca^{2+} sensors. Third panel illustrates the identification of CaM or any Ca^{2+} sensor targets. Fourth panel indicates screening the proteome chip with a target to identify its sensors and other interacting proteins.

ham-Skelton et al., 1994; Fromm and Chua, 1992; Kim et al., 2002b; Reddy et al., 2002; Snedden and Fromm, 2001; Yang and Poovaiah, 2003; Zielinski, 2002b). The data from these studies indicate that CaM interacts with divergent groups of proteins in a Ca^{2+}-dependent and -independent manner (Table 4). Ca^{2+}-CaM interacting proteins identified so far in plants are proteins implicated in generating Ca^{2+} signatures (Ca^{2+}-ATPases and CNG-channels), metabolic or signaling enzymes (glutamate decarboxylase, ATPase, glyoxalase, peptidyl prolyl isomerase (PPI), apyrase, catalase, nitricoxide synthase), phosphorylation and dephosphorylation signaling (kinases including C/CaMK and CaMKs; phosphatases (PP7, MKP1, DsPTP1 and protein phosphatase 2C), stress-induced processes (ACBP60s, CB-HSPs, HSP70s), salt tolerance (glyoxalase, chaperonin), transporters (MDR-like), hormone signaling (SAURs), disease resistance (MLO), cytoskeletal motor (KCBP), translational processes (ribosomal L19 and EF1α), transcriptional regulation (EICBPs/AtSRs/CAMTAs, and TGA3), developmental processes (receptor-like kinases such as CLV1 and SRK) and some not yet identified functions (hypothetical proteins) (Table 4). CaM interacts with myosins in a Ca^{2+}-independent manner and regulates their activity. Although the roles of myosins in transportation, signaling, cell division and morphogenesis and cytoplasmic streaming are known, the involvement of CaMs in a given processes is not well defined (Reddy and Day, 2001).

Comparative studies of CBPs between plants and animals revealed that although CaM is conserved, they differ in many Ca^{2+}-CaM-mediated signaling processes. First, although both have some common CBPs, plants and animals have a unique set of proteins. Second, plants have more paralogs of CBPs and they are expressed differentially (Ali et al., 2003; Reddy et al., 2002; Snedden and Fromm, 2001; Zielinski, 1998). Third, some proteins are present in both animals and plants but the CBD is present only in either plant or animal proteins (Reddy et al., 2002). For example, GADs and catalases in plants bind CaM but not their animal counterparts (Baum et al., 1993; Baum et al., 1996; Yang and Poovaiah, 2002b). Similarly, animal Na^+/H^+ exchanger and p68 RNA helicase are CaM-binding proteins but not in plants. Fourth, although both plants and animals contain CNGCs and Ca^{2+}-ATPases, they differ in either the location of the CBD (C terminal CBD in plant and N terminal CBD in animal CNGCs) and/or functional aspects/regulation. For instance, (i) plant CNGCs are implicated in mediating abiotic and biotic responses (Clough et al., 2000; Demidchik et al., 2002a; Talke et al., 2003) whereas animal CNGCs are involved in olfactory signaling (Liu et al., 1994), (ii) Ca^{2+} pumps in plants and animals may be differentially activated in response to various stimuli, (iii) a plant CaM-binding motor (KCBP) regulates plant-specific activities such as trichome morphogenesis and cell-division-associated activities (phragmoplast formation) whereas these activities are absent in sea urchin which contains a CaM-binding kinesin-C (Bowser and Reddy, 1997; Oppenheimer et al., 1997; Rogers et al., 1999; Vos et al., 2000). Thus the CBP analysis indicates a remarkable difference in Ca^{2+}-CaM-mediated signaling cascades between plants and animals

2.3.2. *Ca^{2+}-salt-overlay-sensitive/calcineurin B-like interacting protein kinases*

The CBL-interacting proteins were identified primarily using yeast two-hybrid assays. Unlike CaM that interacts with divergent proteins, CBLs seem to interact with a specific set of proteins, namely SOS-interacting protein kinases or CBL-interacting protein kinases (SIPK/CIPK) (Guo et al., 2002; Halfter et al., 2000; Liu et al., 2000; Luan et al., 2002; Zhu, 2003). The N- and C-termini of CIPKs determine the specificity with CBLs and all three EF-hand motifs of CBLs are required for interaction with CIPKs (Kim et al., 2000; Shi et al., 1999). For example, SOS3/CBL4 interacts with a SOS2/SIPK24 and which in turn regulates a Na^+/H^+ exchanger (SOS1) and confers salt tolerance in *Arabidopsis*. There are 25 SIPKs/CIPKs in the *Arabidopsis* genome (Table 4). Further studies indicate some CBLs interact with multiple SIPKs/CIPKs and vice versa and some CBLs may not interact with any CIPKs suggesting CBLs might interact with other proteins (Kim et al., 2000). Therefore, it is hypothesized that various CBL/CIPK matching pairs might be mediating stress-related processes through Ca^{2+} signaling.

2.3.3. *Ca^{2+}-dependent protein kinase substrates*

CPKs are the major group of plant-specific (except some protozoans) Ca^{2+} sensors that possess a Ca^{2+}-dependent kinase activity. The CPKs are involved in mediating developmental, hormone and abiotic/biotic stress-induced cellular responses in plants (Harmon, 2003; Harmon et al., 2000; Ludwig et al., 2004; Sheen, 1996). In vitro phosphorylation studies indicate that CPKs interact with a variety of divergent proteins as is the case of CaM targets (Table 5). CPKs phosphorylate proteins that are involved in nitrogen and carbon metabolism (Douglas et al., 1998; McMichael et al., 1995a; McMichael et al., 1995b; Nakai et al., 1998), defense-related processes (Cheng et al., 2001; Romeis et al., 2000), protein degradation (Neumann et al., 1994; Neumann et al., 1996), cytoskelatal organization (Allwood et al., 2001; McCurdy and Harmon, 1992) and ABA signaling processes (Li et al., 1998). Further, the transcriptional and kinase activity of certain CPKs have been shown to be increased in response to phytohormones (GA, BL) drought, ABA and cold suggesting the requirement of CPK kinase activity in these processes (Abo-el-Saad and Wu, 1995; Sheen, 1996; Yang and Komatsu, 2000).

Table 4
Identified CaM and CBL targets and their possible function/activity (a complete list of proteins are available at: www.arabidopsis.org/info/genefamily/CBP.html) Reddy et al. (2002)

Target[a]	Domains	M[b]	Function/activity	Reference
CaM targets or CaM-binding proteins (CBPs)				
1. GADs	Decarboxylase	5	GABA signaling	Baum et al. (1993), Baum et al. (1996)
2. Catalase	Catalase	3	Removal of H_2O_2	Yang and Poovaiah (2002b)
3. MLO	TM	15	Defense against mildew in barley	Kim et al. (2002a), Kim et al. (2002b)
4. Hypothetical	–	2	Not known	Reddy et al. (2002)
5. Hypothetical	–	1	Not known	Reddy et al. (2002)
6. Hypothetical	NLS and unknown repeats	1	Not known	Reddy et al. (2002)
7. Hypothetical	RING	2	Not known	Reddy et al. (2002)
8. SAURs	–	5	Auxin signaling	Yang and Poovaiah (2000b)
9. ACBP60s	–	7	Stress tolerance?	Lu and Harrington (1994), Reddy et al. (1993)
10. TGA3	DNA binding domain	4	Transcription factor	Miao et al. (1994), Szymanski et al. (1996)
11. NPG1	TPRs	3	Pollen germination	Golovkin and Reddy (2003), Safadi et al. (2000)
12. CB-HSPs	–	3	Thermotolerance?	Lu et al. (1995)
13. C/CaMK[c]	Kinase	–	Phosphorylation	Levy et al. (2004), Liu et al. (1998), Mitra et al. (2004), Patil et al. (1995)
			Nodulation	
14. LRR-receptor kinases[d]	LRR and kinase	4	Developmental processes	Vanoosthuyse et al. (2003), Charpenteau et al. (2004)
15. KCBP[e]	Kinesin, MyTH4 and talin like	1	Cell division and trichome morphogenesis	Bowser and Reddy (1997), Oppenheimer et al. (1997), Reddy et al. (1996), Vos et al. (2000)
16. CNGCs	cNMP, TM	20	Ionic transport	Kohler et al. (1999)
17. EICBPs (SRs/CAMTAs)	CG1 and IPT/TIG DNA-binding domains, ankyrin motifs	6	Stress tolerance and gene regulation?	Bouche et al. (2002), Reddy et al. (2000), Taleb and Fromm (2004), Yang and Poovaiah (2002a)
			AtCAMTA1 and –5 enhance *AVP1* gene expression	Mitsuda et al. (2003)
18. ATPase		1	Cell division and vesicle fusion	Buaboocha et al. (2001)
19. Glyoxalase I and II		1	Salt tolerance	Singla-Pareek et al. (2003), Veena et al. (1999)
20. Apyrase		1	Light signaling and ATP transport	Hsieh et al. (1996)
21. Chaperonin		1	Salt tolerance	Yang and Poovaiah (2000a)
22. MDR-like	TM	1	Transport	Wang et al. (1996)
23. PP7	–	1	Phosphatase activity	Kutuzov et al. (2001)
24. HSP-70s	–	6	Thermotolerance	Sun et al. (2000)
25. ACAs[f]	TMs	13	Ca^{2+} efflux	Geisler et al. (2000), Harper et al. (1998)
26. PPI	–	2	Functions in pollen development	Vucich and Gasser (1996)
27. EF-1α	–	4	Translational regulation	Moore et al. (1998)
28. Ribosomal L19	–	3	Not known	Sonnemann et al. (1991)
29. NtMKP1 (tobacco CBP)	Kinase, Gelsolin, and Ser-rich	1	Wound and defense signaling	Yamakawa et al. (2003)
30. DsPTP1	Pohsphatase	1	Dephoshorylation of (phospho S, T, Y)	Yoo et al. (2003)
31. Protein phosphatase 2C (PCaMPP)	Phosphatase	1	Dephoshorylation	Takezawa (2003)
32. Nitricoxide synthase	Enzyme	1	Production of NO	Guo et al. (2003)
33. CaMK	Kinase	3	Phosphorylation	Zhang and Lu (2003)
34. Myosins	Myosin motor domain, IQs	17	Cytoskeletal functions	Reddy and Day (2001)

Table 4 (*continued*)

Target[a]	Domains	*M*[b]	Function/activity	Reference
CBL sensor targets				
SIPK/CIPK (CBL interacting protein kinases)	Kinase domain	25	Salt tolerance and possibly other stress tolerance mechanisms	Cheong et al. (2003), Guo et al. (2001b), Shi et al. (1999)

[a] The animal homologs to plant GAD and catalases do not bind CaM. CBPs 3-14 in this column are specific to plants. CBPs 16-34 are present in plants and animals. Myosins interact with CaM in a Ca^{2+}-independent manner.

[b] Number of family members (M).

[c] C/CaMK homolog in *Arabidopsis* is yet to be identified.

[d] Members include SRK, AtCaMRLK, AtRLK4 and CLV1 are shown to bind CaM in a Ca^{2+}-dependent manner; Vanoosthuyse et al. (2003), Charpenteau et al. (2004).

[e] Two sensors (CaM and KIC) interact with KCBP. A kinesin that binds to CaM has been reported in sea urchin but MyTH4 (myosin tail homology 4) and talin-like domains are absent in sea urchin kinesin-C.

[f] ACA2 is regulated differentially by CaM (positive) and CPK (negative).

Table 5
Identified CPK sensor targets

Substrate	CPK source	Function/activity	Reference
Nitrate reductase	Spinach, AtCPK3	Nitrogen metabolism	Douglas et al. (1998), McMichael et al. (1995a)
Sucrose phosphate synthase	Spinach	Carbon metabolism	McMichael et al. (1995a), McMichael et al. (1995b)
Sucrose synthase	Soybean	Carbon metabolism	Nakai et al. (1998), Zhang and Chollet (1997)
Phosphoenolpyruvate carboxylase	Maize and soybean	Carbon metabolism	Ogawa et al. (1998)
Phenylalanine ammonia lyase	French bean and At1g18890	Secondary metabolite synthesis	Cheng et al. (2001)
Pseudoresponse regulator	CPK1 from Ice plant	Stress response	Patharkar and Cushman (2000)
Carboxypeptidase inhibitor	Wheat	Antifungal	Neumann et al. (1996)
Protease inhibitor	Wheat	Antifungal	Neumann et al. (1994)
ACA2	At4g37640	Ca^{2+} pump	Hwang et al. (2000b)
Aquaporins	Spinach	Water transport	Huang et al. (2001)
PM H^+-ATPase	Oat and maize	Ion transport	Camoni et al. (1998), Harmon et al. (1996)
KAT1	*Vicia faba*	Potassium transport	Li et al. (1998)
Actin depolymerizing factor	French bean	Cytoskelatal regulation	Allwood et al. (2001)
CPK-related kinase	Carrot DcCPK1	Phosphorylation	Farmer and Choi (1999)
Myosin light chain	*Chara*	Cytoskelatal regulation	McCurdy and Harmon (1992)
Proteasome regulatory subunit	NtCPK1	Protein degradation	Lee et al. (2003)
Components in Cf9/Avr9-induced HR	NtCPK2 and 3	Defense response	Romeis et al. (2001)
Ser acetyltransferase	Soybean	RNase activity	Harmon (2003)

Although there are 142 *Arabidopsis* proteins with C2 domains, the interacting partners of only two proteins have been identified. These include *Arabidopsis* BONZAI 1, a C2-domain-containing protein (also known as copine), which interacts with BAP1 (BONZAI 1-associated protein 1) (Hua et al., 2001) and tobacco PLDδ interacts with microtubules and plasma membrane in a Ca^{2+}-dependent manner (Gardiner et al., 2001).

2.3.4. Ca^{2+} sensor–target paradigm

The Ca^{2+} sensor-target analysis indicates that different sensors interact with different sets of targets. CaMs and CPKs interact with a wide variety of targets whereas CBLs interact with a unique set of protein kinases, CIPKs/SIPKs. Targets for many other Ca^{2+}-sensors remain to be identified in *Arabidopsis* and other plants (Table 3). Interestingly, there are instances in which a target is regulated by two sensors. For example, KCBP interacts with CaMs and KIC, a Ca^{2+}-binding protein with one EF-hand motif. Although both sensors inhibit interaction of KCBP with microtubules, KIC requires a lower concentration of Ca^{2+} than CaM (Reddy et al., 2004). *Arabidopsis* Ca^{2+}-ATPase (ACA2) is also known to interact with CaM and CPK. The activity of ACA2 is regulated differentially by CaM (positive) and CPK (negative) (Hwang et al., 2000a,b). CaM sensors differentially regulate the targets involved in a process. For example, CaM activates catalase and inhibits NADPH oxidase activities and both targets are involved in removal and synthesis of H_2O_2, respectively (Yang and Poovaiah, 2002b). Similarly, CaM activates Ca^{2+}-ATPase and inhibits CNGC activities (Hwang et al., 2000a; Kohler et al., 1999). These possibilities of interactions between a sensor and multiple targets and differential

regulation of targets by sensors at varied Ca^{2+} concentrations point to a complex network of sensor–target interactions in Ca^{2+}-regulated cellular processes. Alternatively, some of the interactions between sensor and target (CaM:CBPs, CBL:SIPK/CIPK or CPK:targets) may not truly reflect in vivo interactions since most of these interaction studies were carried out using the yeast two-hybrid or in vitro protein–protein interaction-based techniques.

3. Power of proteomics in identifying Ca^{2+} signaling components

Although many Ca^{2+} signaling components have been identified recently, it is necessary to identify all components and interaction between the components. Recent advances in high-throughput technologies using protein and DNA chips offer novel approaches to address this problem and to elucidate all the networks in the Ca^{2+}-regulated grid. As an *Arabidopsis* ORFeome (collection of all full-length cDNAs) is becoming available, bacterial expression of the cDNAs as fusions to a tag (His/GST) and purification in a high-throughput (HT) manner are being developed. Hence, in the near future it will be possible to array all of the ~30,000 proteins on a solid surface as in the case of yeast (Zhu et al., 2001). As shown in Fig. 2b, a protein microarray chip with all *Arabidopsis* proteins could be very useful in genome-wide identification of Ca^{2+} signaling components. Recently, RGS-His_6-tagged fusions of 95 *Arabidopsis* ORFs were constructed, expressed, purified, robotically arrayed on glass sides (nitrocellulose based polymer or polyacrylamide) and used to detect as low as 0.1 fmol/spot with an anti RGS-His_6-tag antibody (Kersten et al., 2003). Further, this 95-protein chip was successfully used to detect specific proteins using corresponding antibodies. Studies indicate that large size rather than small size tags yield more stable and higher yield proteins (e.g., GST tag versus His tag) (Braun et al., 2002). Recently, GST-fusion proteins of the 6000 ORFeome of yeast were successfully purified, arrayed and screened with biotinylated CaM in the presence of Ca^{2+} (Zhu et al., 2001). As the *Arabidopsis* genome is 6X bigger than yeast, it is possible that all the *Arabidopsis* ORFeome can be cloned as GST fusions, expressed in a HT manner and robotically arrayed on a solid surface (Fig. 2b). The presence and amount of protein on each spot can be calibrated by probing with GST antibody (Fig. 2b, top panel) as a prerequisite prior to using the chip for identification of targets and affinities between sensors and targets. These chips can then be used for comprehensive analysis of all Ca^{2+} signaling components and the interactions among them in a very efficient way.

The *Arabidopsis* proteome chip can be used in a number of ways to study Ca^{2+} signaling networks. First, the protein chip can be probed with a $^{45}Ca^{2+}$ ligand to: (1) test whether all EF-hand motif containing proteins bind Ca^{2+}, (2) identify other Ca^{2+} sensors without EF-hand motifs that bind Ca^{2+} and (3) to differentiate sensor affinity to Ca^{2+}. After probing with $^{45}Ca^{2+}$, the intensity of spots on the chip can be scanned with a phosphoimager and be quantitatively calculated (Fig. 2b). Second, the proteome chip can be probed with labeled (biotinylated) CaM or any other Ca^{2+} sensor in the presence of Ca^{2+}. Using this approach, screening of a yeast proteome chip containing 5800 ORFs with biotinylated CaM resulted in identification of 39 (including many unknown) CBPs (Zhu et al., 2001). The interaction between the CBP and biotinylated CaM probe on the hybridized chip is detected with Cy3-labeled streptavidin using a control chip probed with Cy3-labeled streptavidin alone.

Third, as in the case of yeast kinases (Zhu et al., 2000), the entire proteome-chip of *Arabidopsis* can be used to screen for substrates using a recombinant CPK in the presence of (γ-^{32}P)ATP and Ca^{2+} and the phosphoprotein can be detected with a phosphoimager (display not shown). Because of the presence of a large number of kinases in the *Arabidopsis* genome as well as autophosphorylation of CPKs it is necessary to have several controls. Alternatively, a proteome display method can be used to identify all substrates of a protein kinase as described (Corcoran et al., 2003).

Fourth, the major advantage of using an HT-proteome chip in Ca^{2+} signaling is to identify and quantify interactions between a given target and all sensors at a time. Although a quantification assay has not yet been developed to assay interaction between proteins, 30% of 115 antibody:antigen pair interactions of animal origin were successfully quantified (Haab et al., 2001). To further improve this quantification method on an HT scale, two antibodies against different regions of the same antigen were developed. In this assay, the first AB is immobilized to a solid surface, detected by antigen which in turn is detected by probing with a second AB (sandwich assay: ab–ag–ab). Using the sandwich assay, the interaction between a protein of interest (sensor or target of Ca^{2+} signaling) and the entire proteome could be quantified using a His-tag fused probe (sandwich assay—protein:His-tagged protein probe:His-tag.AB). This assay not only resolves possible interactions between sensors and targets but also quantifies the extent of interactions (Fig. 2b). Another puzzle in Ca^{2+} signaling concerns the true interactions between sensors and targets at physiological levels. This could be resolved by probing a proteome chip containing equimolar concentrations of proteins with varying concentrations of Ca^{2+} sensor. The analysis would yield strong to weak targets for a given probe.

Although the HT-based protein–ligand or protein–protein interaction screens would provide invaluable

information towards understanding their interactions, there are limitations. For example, the conformation of protein on the chip, accessibility to probe, lack of post-translational modifications or partnership in a complex.

4. Tandom-affinity purification: identification of proteins in a functional complex

The cellular activities within cells or tissues are likely to be coordinated by multiple combinations of interactions among the proteins. In addition, sessile plants have to cope with unexpected adverse environmental factors. Therefore, proteins often form complexes to regulate cellular processes in a "demand–supply" fashion. For example, although the SOS2 protein is known to interact with SOS1, a plasma membrane Na^+/H^+ exchanger (Qiu et al., 2002), it also interacts with other proteins involved in different cellular activities such as protein phosphatase 2C (ABI2) (Ohta et al., 2003) and H^+/Ca^{2+} antiporter CAX1 (Cheng et al., 2004). This may apply to other Ca^{2+}-sensor targets. To isolate interacting proteins in plants and other organisms, one-on-one protein–protein interaction-based screening such as the yeast two-hybrid assay was extensively used. However, using this method, it is not possible to isolate protein complexes that comprise several proteins. Because the Ca^{2+}-mediated processes are very dynamic in nature and because of multiple proteins at each "node" of Ca^{2+} signaling, it is likely that the Ca^{2+}-mediated processes involve formation of protein complexes among signaling components and/or with other cellular proteins.

To unravel interacting proteins in Ca^{2+} signaling, the "tandom-affinity-purification" (TAP) method could be effectively used. TAP and its modified version iTAP (using RNAi technology to suppress the endogenous protein for efficient pullout of complexes using introduced bait protein) were used in identifying the composition of biological complexes in yeast, *Drosophila* and human systems (Forler et al., 2003; Gavin et al., 2002; Gavin and Superti-Furga, 2003; Puig et al., 2001; Rigaut et al., 1999). In this method, two affinity tags (either CaM-binding peptide, His or GST tag and a Protein A tag) separated by a TEV protease site are attached to the N- or C-terminus of protein of interest. The Protein A tag is always at the extreme end of the bait protein (Fig. 3). This TAP cassette is introduced into the living system and expressed at a level equal to its endogenous level. Then the cell extracts are processed and purified using IgG beads and the bound proteins are eluted with TEV protease. The eluted protein complex is then passed through a second affinity matrix (CaM, His or GST-Sepharose beads). Proteins in the second elution are treated with trypsin, the masses of resultant peptides will be calculated using MALDI-TOF-mass-spectrometer and/or (LC) nano-ESI-MS/MS. The peptide mass list will then be used to search against protein databases to identify proteins (Chang et al., 2000).

Depending on the bait protein, the second affinity tag can be replaced with His or GST tags and can be purified accordingly. Because CaM influences its target protein association with others both negatively and positively, other tags (His or GST) can be used if a CBP is used as bait in TAP. This is a more effective method than the yeast two-hybrid assay because it allows identification of all proteins that interact with the bait either directly or indirectly in a single step.

5. Functional analysis of Ca^{2+} signaling components in plants

Because Ca^{2+} mediates a wide variety of processes (Table 1) and because of the presence of about 700 components at different "nodes" (Tables 2–5), it is necessary to identify roles of these signaling components. To this end, cell-biological, molecular genetic and transgenic approaches have been extensively utilized to determine the functions of individual components (Table 6). However, because of overlapping and/or redundant functions of various components and their families, genome-wide analysis together with the above approaches is expected to provide valuable information about each component and their interactions (Figs. 2–5).

5.1. Cell-biological approaches

Elevation of $[Ca^{2+}]_{cyt}$ levels in response to a variety of stimuli has been well documented using cell biological approaches (Table 1). Cellular origin of Ca^{2+} signatures in response to wind and cold was analyzed through localized expression of aequorin (cytoplasm MAQ 2.4; and nucleus MAQ 7.11) coupled with monitoring of *NpCaM1* gene expression (van Der Luit et al., 1999). Cameleon indicators also allowed measurement of $[Ca^{2+}]_{cyt}$ levels in guard cell signaling (Allen et al., 1999; Scrase-Field and Knight, 2003). Reporter genes (e.g., GUS, GFP, LUC, CAT) under the control of a promoter regulated by Ca^{2+} signaling components are widely used in cell biological approaches to decipher the effect of input signals (Table 6). In a transient assay using an osmotic stress responsive gene promoter fused to luciferase (HVA1-LUC) reporter system in maize protoplasts, the role of two of the eight tested *Arabidopsis* CPKs in abiotic stress-responsive pathways has been determined (Sheen, 1996). The role of H_2O_2-mediated Ca^{2+} influx and CPK-dependent phosphorylation in pathogen and elicitor induced hypersensitive response (HR) development has also been proved using a soybean cell culture system (Levine et al., 1996). Functional studies with soybean conserved and divergent CaMs

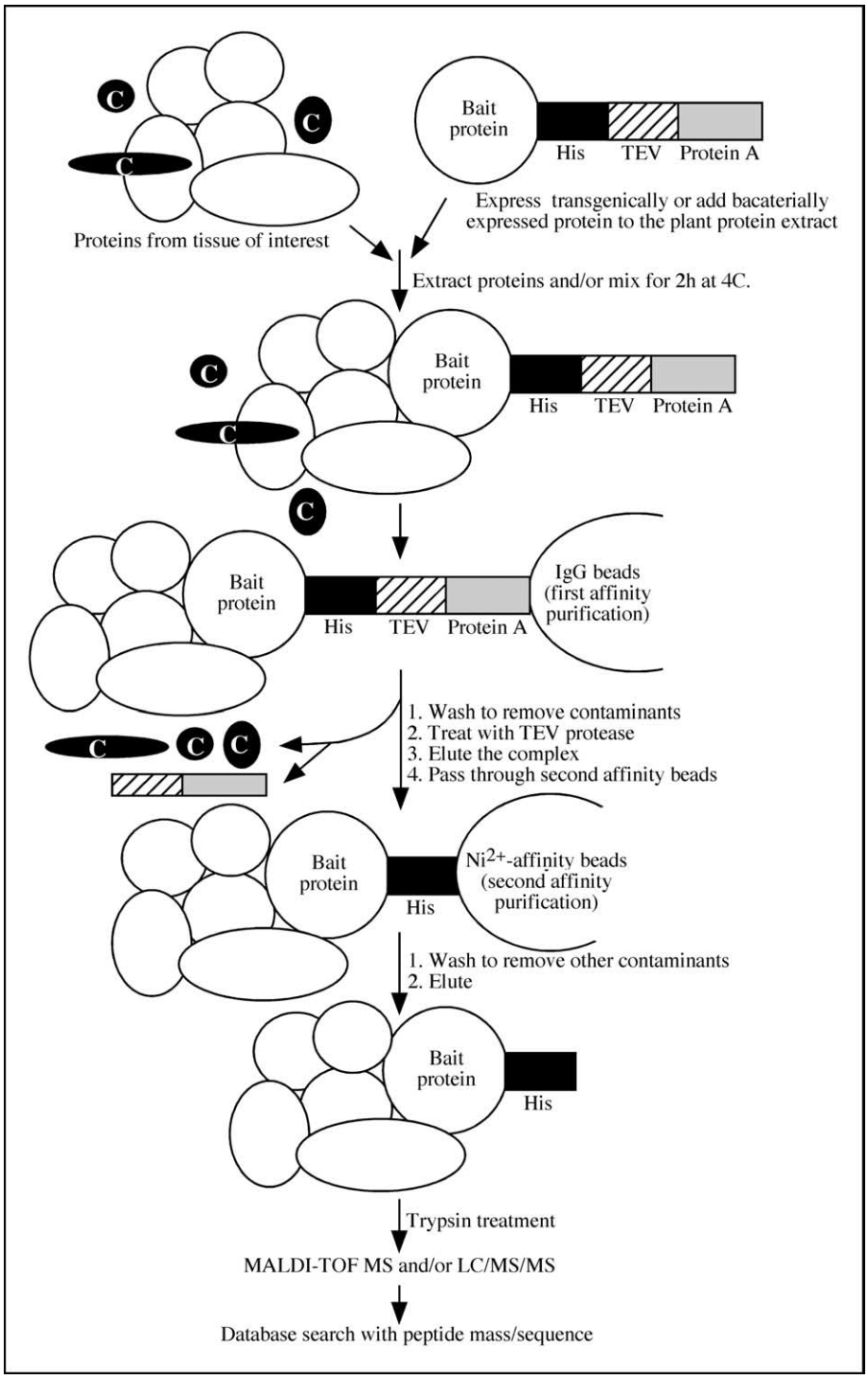

Fig. 3. Schematic representation of tandom affinity purification (TAP) method for isolation of in vivo protein complexes. The bait protein is tagged with two affinity tags (His and protein A at the C-terminus) and the TEV protease cleavage site in the middle. This recombinant protein is either mixed with cell extract or expressed in vivo with the promoter of the bait protein. The protein complex associated with bait protein is isolated by passing through IgG beads, washing the column and eluting with TEV protease. These proteins are passed through a Ni^{2+} affinity column and pure protein complex is eluted with appropriate buffer. The eluted proteins is subjected to trypsin treatment and the resultant peptide mass is estimated by MALDI-TOF-MS and/or LC/MS/MS. C, contaminant proteins. (modified from Puig et al. (2001)).

have revealed that they differ in their affinity and/or target preference and divergent CaMs participate in disease resistance (Heo et al., 1999; Lee et al., 1995; Lee et al., 1999; Lee et al., 1997). Involvement of a CaM target, KCBP, a microtubule binding motor, in plant-specific cell division processes has been determined using immunolocalization and injection of KCBP specific antibodies in cultured cells and *Tradescantia* stamen hair

Table 6
Functional analysis of the Ca^{2+} signaling components

Gene product	Approach[a]	Function/activity	Reference
Cell-biological approach			
Arabidopsis CPK 10 and CPK 11	Transient assay	Drought and salt-induced gene expression	Sheen (1996)
Arabidopsis CPK30	Transient assay	Cold, salt and ABA induced gene expression	Sheen (1996)
KCBP/ZWICHEL	Immuno-localization and injection of antibodies	Associated with plant-specific cell division processes	Bowser and Reddy (1997), Vos et al. (2000)
Molecular genetic approach			
SOS3/SOS2/SOS1	EMS or T-DNA mutants	Salt tolerance	Qiu et al. (2002), Zhu (2002)
KCBP/ZWICHEL	EMS	Trichome branching	Hulskamp et al. (1994)
Transgenic approach			
Glutamate receptor 2 (AtGLR2)	OE in *Arabidopsis*	Ca^{2+} utilization	Kim et al. (2001)
At Ca^{2+}/H^{+} antiporter 1 (CAX1)	OE in tobacco	Ion imbalance, stunted growth, sensitive to cold	Cheng et al. (2003a), Hirschi (1999)
At Ca^{2+}/H^{+} antiporter 3 (chimeric protein with 9 aa from CAX1)	OE in tobacco	Ion imbalance, stunted growth, sensitive to cold	Shigaki et al. (2002)
Yeast Ca^{2+}/H^{+} antiporter	OE in *Arabidopsis* and tobacco	Ca^{2+} efflux carrier	Hirschi et al. (2001)
Medicago truncatula 1 (Annexin)	Gene expression and promoter:GFP or GUS reporter	Induced by Nod factors and involved in nodulation	De Carvalho-Niebel et al. (2002)
Pea PPF1 Ca^{2+} ion carrier	OE in *Arabidopsis*	Inhibits programmed cell death (PCD)	Li et al. (2004)
Arabidopsis putative Ca^{2+} transporter (PPF1)	OE in *Arabidopsis*	Flowering time	Wang et al. (2003b)
Arabidopsis CPK1	OE in tomato	Increased NADPH oxidase activity	Xing et al. (2001)
At CRT/DRE-binding transcription factor	OE in tobacco	COR gene expression	Jaglo-Ottosen et al. (1998)
At DRE-binding factor 1A	RD29A driven expression in tobacco	*COR* gene expression	Kasuga et al. (1999)
Soybean CaM4 isoform	OE in tobacco	PR gene expression and biotic stress tolerance	Heo et al. (1999)
Soybean CaM5 isoform	OE in tobacco	PR gene expression and biotic stress tolerance	Heo et al. (1999)
Mutated form of synthetic CaM VU-3 (K115R)	OE in tobacco	Activation of NAD kinase and production of ROSs	Harding et al. (1997)
Petunia hybrida CaM53 isoform	OE in tobacco and *Arabidopsis*	Importance of prenylation domain and cellular localization	Rodriguez-Concepcion et al. (1999b)
Tobacco regulator of gene silencing-CaM-like protein (rgs-CaM)	OE in tobacco	Suppresses posttranscriptional gene silencing	Anandalakshmi et al. (2000)
Arabidopsis KCBP interacting Ca^{2+}-binding protein	OE in *Arabidopsis*	Trichome stalk length and branching (regulator of KCBP)	Reddy et al. (2004)
Arabidopsis Calcineurin B-like1	OE in *Arabidopsis* with MAS super promoter	Enhanced tolerance to salt and drought and sensitive to cold	Albrecht et al. (2003), Cheong et al. (2003)
Arabidopsis Ca^{2+} sensing receptor (low affinity, high capacity Ca^{2+}-binding protein:CAS)	OE in *Arabidopsis*	Guard cell signaling and impairs bolting	Han et al. (2003)
Rice CPK7	OE in rice	Cold and salt stress	Saijo et al. (2001)
Rice CPK2	OE in rice	Disruption of seed development	Morello et al. (2000)
Maize Ca^{2+}-binding protein (Calreticulin)	Heat shock promoter driven expression in *Arabidopsis*	Increases Ca^{2+} stores in ER	Persson et al. (2001), Wyatt et al. (2002)
Bj Glyoxalase I and II	OE in tobacco	Tolerance to NaCl, methyglyoxal	Singla-Pareek et al. (2003), Veena et al. (1999)
Petunia glutamate decarboxylase	OE in tobacco	Synthesis of GABA and stress tolerance	Baum et al. (1996)
Membrane-bound cation channel NtCbp4	OE in tobacco	Ni^{2+} tolerance	Arazi et al. (1999)
Barley MLO	Transient assays in barley	Defense against mildew in barley	Kim et al. (2002b)
Wheat FKBP73 (PPI)	OE in rice	Pollen development	Kurek et al. (2002)

(*continued on next page*)

Table 6 (*continued*)

Gene product	Approach[a]	Function/activity	Reference
Nitricoxide synthase (AtNOS1)	OE in *Arabidopsis*	Overproduction of nitric oxide and hormonal signaling	Guo et al. (2003)
Knockout approach			
Maize pollen CPK	Antisense oligonucleotides	Inhibition of pollen germination	Estruch et al. (1994)
Tobacco CPK 1	Virus-induced gene silencing	Constitutive defense activation and defects in cell division and differentiation	Lee et al. (2003)
Arabidopsis KCBP	T-DNA insertion	Trichome stalk length and branching	Oppenheimer et al. (1997)
Arabidopsis ECA 1	T-DNA insertion	Tolerance to Mn^{2+}	Wu et al. (2002)
Arabidopsis ABC transporter (AtMRP5)	T-DNA insertion	Guard cell signaling	Klein et al. (2003)
Arabidopsis NPG1	T-DNA insertion	Essential for pollen germination	Golovkin and Reddy (2003)
Arabidopsis CNGC2	T-DNA insertion	Vegetative and reproductive growth, programmed cell death, and adaptive responses to biotic and abiotic stimuli	Chan et al. (2003)
Arabidopsis RbohD and F	T-DNA insertion	ROS production and ABA-mediated signaling in guard cells	Kwak et al. (2003)
CIPK14/AtSR2	T-DNA insertion	Sugar sensing mechanism	Chikano et al. (2001)
Arabidopsis CBL interacting protein 3	T-DNA insertion	ABA and cold signal transduction	Kim et al. (2003b)
AtPLC1	Antisense	Insensitive to ABA-mediated seed germination and growth	Sanchez and Chua (2001)
AtPLDα	Antisense	Retards ABA- and ethylene-promoted senescence	Fan et al. (1997), Sang et al. (2001)
AtPLDβ	Antisense	Reduced production of PA in response to dehydration	Katagiri et al. (2001)
DEK1	*Mutator* transposon insertion	Essential for maize grain aleurone cell development	Lid et al. (2002), Wang et al. (2003a)
BON1/CPN1	T-DNA insertion	Humidity and temperature associated plant growth and development	Hua et al. (2001), Jambunathan et al. (2001)

[a] OE, overexpression of genes under CaMV 35S promoter.

cells (Bowser and Reddy, 1997; Vos et al., 2000). Although accumulating evidence indicate roles of Ca^{2+}, sensors (CaMs and CPKs) or their targets in abiotic and biotic stress responses (Table 6), identification of intermediate components, for example, *HVA*1 target genes in osmotic stress, CPK substrates in HR and divergent SCaM isoform-specific targets in disease resistance have not been identified.

5.2. Molecular genetic approaches

Mutants that show an altered response to a particular stress are invaluable resources in identifying components of signal transduction pathway(s). The best studied Ca^{2+} signaling pathway is the SOS mediated potassium and sodium ion balance during the salt tolerance process in *Arabidopsis*. This elegant study began with assaying EMS or fast-neutron mutagenized M2 seed or T-DNA insertion *Arabidopsis* lines (~267,000) in a salt overly sensitive (SOS) assay, which resulted in the discovery of three genes namely, *SOS3*, *SOS2* and *SOS1*. Further biochemical and molecular studies revealed a linear Ca^{2+} signaling circuit that consists of SOS3 (a Ca^{2+} sensor) → SOS2 (a protein kinase) → SOS1 (a Na^+/K^+ transporter that mediates salt tolerance) (Cheng et al., 2004; Epstein, 1998; Liu and Zhu, 1997, 1998; Qiu et al., 2004; Wu et al., 1996; Zhu et al., 1998) (Table 6). Functional characterization of plant genes in yeast mutants or vice versa is also a helpful tool for characterization of Ca^{2+} signaling genes. Constitutive expression of yeast calcineurin subunits in tobacco enhanced salt tolerance (Pardo et al., 1998) and a yeast Ca^{2+}/H^+ antiporter in *Arabidopsis* conferred Ca^{2+} efflux activity (Hirschi et al., 2001). Similarly, complementation of yeast mutants by plant Ca^{2+}-pumps (ACA2 and ECA1) confirmed the existence of similar functional Ca^{2+} machinery between yeast and plants (Harper et al., 1998; Hwang et al., 2000a).

Molecular and genetic approaches led to identification of a set of genes *COR* (cold responsive) that respond to cold, drought and ABA which are well-known stress factors that elevate $[Ca^{2+}]_{cyt}$ (Table 1) (Hughes

and Dunn, 1996; Knight, 2000; Thomashow, 1998). It is believed that *COR* regulon (*COR6.6, COR15a, COR78, WCS19, CORa, CAS15a, COR47* and *HVA1*) and their transcription regulator CBF (CRT/DRE binding factor) promote tolerance to freezing by regulating enzymes that produce lipids, sugars and other membrane stabilizing components during acclimation (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). The mutant *sfr6* (sensitive to freezing 6) showed a wild-type Ca^{2+} sensing system and CBF (*CBF1-3*) gene expression but showed reduced transcript levels for some of the *COR* regulon genes (*KIN1, COR15a,* and *LTI78*) in response to cold, suggesting a missing link between CBF and the *COR* regulon (Boyce et al., 2003; Knight et al., 1999). Several other mutants have been extensively used in dissecting the complex network of stress- and developmental-signaling in plants (Allen and Schroeder, 2001; Ishitani et al., 1997).

5.3. Overexpression or loss-of-function analyses: possible interactions between sensors and targets

To address the functions of Ca^{2+} signaling components at the whole plant level, transgenic plants with genes encoding Ca^{2+} channels, sensors and targets were generated and their phenotypes were evaluated in developmental processes and/or in response to stress signals (Table 6). Constitutive expression of AtGLR2, antiporter (*CAX1*), annnexins and Ca^{2+} carriers (PPF1) either in tobacco or *Arabidopsis* revealed their roles in Ca^{2+} homeostasis (De Carvalho-Niebel et al., 2002; Hirschi, 1999; Hirschi et al., 1996; Kim et al., 2001; Li et al., 2004; Wang et al., 2003b).

Arabidopsis, tobacco and rice transformed with CPK genes exhibited tolerance to drought and salt (AtCPK10 and 11) or cold, salt and ABA (OsCPK7), increased NADPH oxidase activity (AtCPK1) or showed disruption in seed development (OsCPK2) (Morello et al., 2000; Saijo et al., 2000; Sheen, 1996; Xing et al., 2001). Although CaMs are conserved, their constitutive expression in transgenic plants revealed specificity in their function. A mutated VU-3 (K115R) isoform showed hyperactivation of NAD kinase and increased production of reactive oxygen species (ROSs) (Harding et al., 1997). Transgenic plants with SCaM4 and SCaM5, but not SCaM1-3, exhibit constitutive expression of SA related gene expression independent of SA throughout their life cycle and enhanced disease resistance to a wide spectrum of pathogens (*Phytophthora parasitica* var *nicotiana, Pseudomonas syringae* pv. *tabaci* and an virulent viral pathogen, TMV) (Heo et al., 1999). Transgenic plants with other Ca^{2+} sensors such as *MAS* promoter-*CBL*1, *CaMV 35S*-CAS, and heat shock promoter-calreticulin also showed tolerance to salt/drought, guard cell signaling and storing Ca^{2+} to ER, respectively (Albrecht et al., 2003; Cheong et al., 2003; Han et al., 2003; Persson et al., 2001; Wyatt et al., 2002). Overexpression of KIC, a Ca^{2+} sensor exhibited defect in trichome morphogenesis that resembles its target (*zwilkcbp*) knockout mutant phenotype suggesting the sensor and the target are in linear Ca^{2+} signaling circuit: $Ca^{2+} \rightarrow$ KIC $\rightarrow$ KCBP $\rightarrow$ microtubule organization $\rightarrow$ trichome branching (Reddy et al., 2004).

Transgenic tobacco with transcriptional factors CBF1 or DREB1A under *CaMV35S* or stress inducible promoter (*rd29A-DREB1A*) showed increased expression of their target *COR* genes and significant resistance to cold, drought and salt stress responses as these signals induced $[Ca^{2+}]_{cyt}$ (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). Transgenic expression analysis for CaM target CBPs such as GAD, GLY, CtCBP4, barley MLO and PPI were also analyzed (Table 6). Transgenic plants expressing GAD without the CaM-binding domain (Ca^{2+}-CaM regulation is abolished) contain high and low levels of GABA and Glu, respectively, and showed abnormal cell elongation in stem cortex and parenchyma tissues (Baum et al., 1996). Constitutive expression of *BjGly*1 and *BjGly*II in tobacco reveled their role in conferring tolerance to salt, methyl glyoxal and drought stresses (Singla-Pareek et al., 2003; Veena et al., 1999). Overexpression of genes encoding CBPs confers Ni^{2+} ion tolerance (NtCBP4, a CNGC channel), disease resistance (MLO) and mediates pollen development (PPI) (Arazi et al., 1999; Kim et al., 2002b; Kurek et al., 2002).

T-DNA insertion, antisense and viral-induced gene knockout approaches were also used to dissect the roles of Ca^{2+} pumps, transporters, Ca^{2+} sensors, CaM-targets and CBL-targets (Table 6). For example, silencing of a Ca^{2+} sensor CPK or the CaM target NPG revealed their essential role in pollen germination. These two independent gene knockout studies suggest one linear Ca^{2+} signaling circuit or two independent circuits: one through CPK and the other through NPG. Gain-of-function, knockout (double or triple mutants in the case of families) and dsRNAi techniques are powerful tools to decipher biological roles of any unknown protein even if it is a member of a family.

Constitutive expression and knockouts of various Ca^{2+} signaling components showed altered responses to developmental, abiotic and biotic-induced cellular processes (Table 6). Further, for proteins of unknown functions with no functional domains, gain- and/or loss-of-function analyses would be a good starting point to gain insights into their role in cellular activities. In addition, targets of different Ca^{2+} sensors in most cellular processes have not been identified. For example, although several Ca^{2+} sensors have been implicated in cold stress response, the Ca^{2+}-mediated signaling circuit in cold-induced gene expression of *COR* genes through CBF transcriptional activity is not identified. NtCPK2 and NtCPK3, but not NtCPK1, are involved in

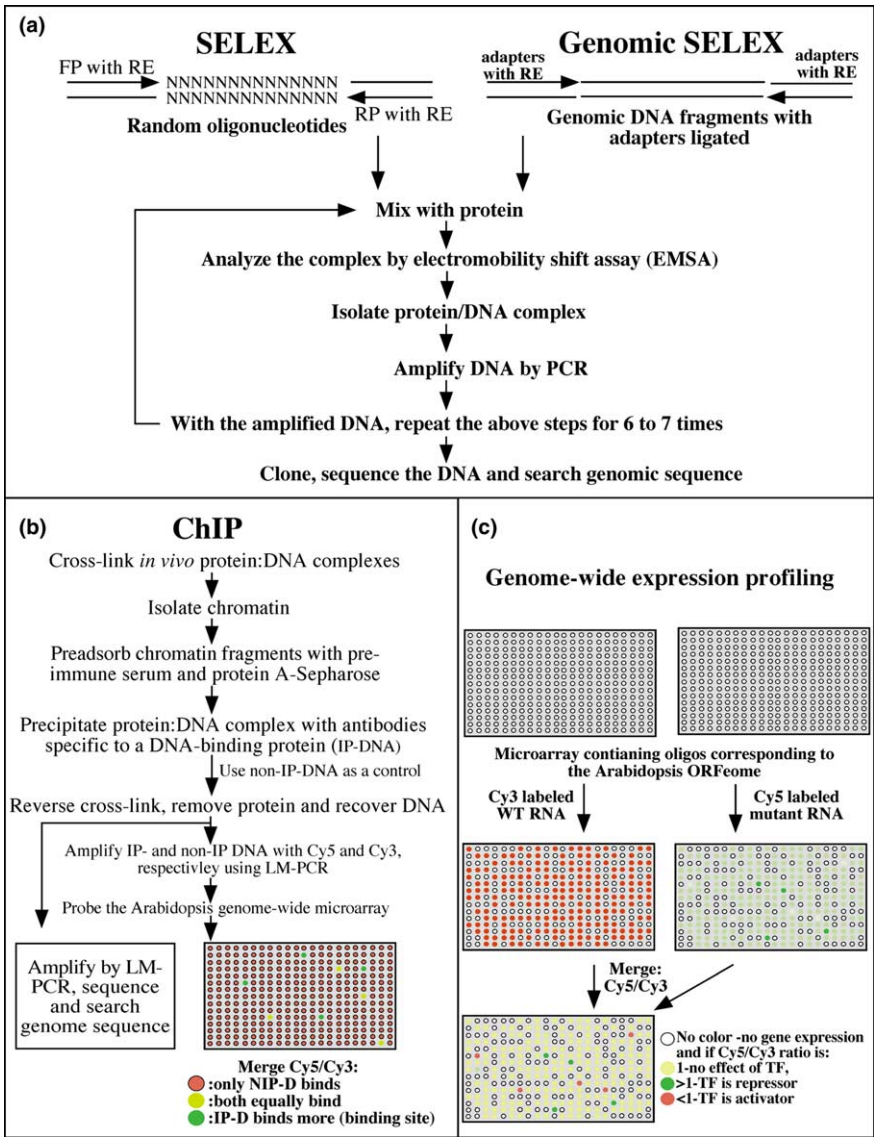

Fig. 4. Genome-wide identification of *cis*-elements and target genes for DNA-binding proteins. (A) Systematic evolution of ligands by exponential enrichment (SELEX) using random oligonucleotides or *Arabidopsis* genomic DNA fragments and purified DNA-binding protein. The oliginuleotides or genomic fragments that interact with the DNA-binding protein can be amplified, cloned, sequenced and used to search genomic sequence database. Using SELEX procedure one can identify putative target genes for DNA-binding proteins. FP, forward primer; RP, reverse primer and RE, restriction enzyme. (B) Chromatin-immuno precipitation (ChIP) assay. The DNA-binding protein:genomic DNA complexes will be precipitated with a specific DNA-binding protein antibody and the DNA from the precipitated complexes (IP-DNA, immunoprecipitated DNA) will be reverse cross-linked, recovered and either sequenced for its identity (ChIP assay) (Johnson et al. (2001); Wang et al. (2002)) or labeled with Cy5 using LM-PCR (control non-IP-DNA will also be labeled with Cy3) and used to screen a genomic microarray with Cy5 IP-DNA and Cy3 non-IP-DNA together (genomic ChIP assay) (Ren et al. (2000)). The spot with higher Cy5 signal contains the *cis*-elements or binding site for the DNA-binding protein. (C). Genome-wide expression analysis. The RNA from wild-type (WT) and a mutant of a DNA-binding protein (or any other mutant of interest at various developmental stages and in response to various stresses) are labeled with Cy3 and Cy5, respectively and use to probe the *Arabidopsis* microarray containing ORF-specific oligos. If the Cy5/Cy3 ratio is less or more than one, then the DNA-binding protein is a putative transcriptional activator or repressor for that particular gene on the spot, respectively. TF, transcriptional factor.

mediating the tobacco *Avr9:Cf-9* defense response pathway via H_2O_2. However, the phosphorylation cascade that couples the Ca^{2+} signaling circuit to the defense response pathway via H_2O_2 is not known. Because NADPH oxidase and catalase are Ca^{2+}-CaM activated targets that are involved in regulation of H_2O_2 biosynthesis and degradation, respectively, one could test whether the *Avr9:Cf-9* defense response pathway is turned on-and-off via $Ca^{2+} \rightarrow CPK \rightarrow NADPH$ oxidase $\rightarrow H_2O_2 \rightarrow$ *Avr9:Cf-9* $\rightarrow ON$ and $Ca^{2+} \rightarrow CaM \rightarrow$ catalase $\rightarrow 2H_2O_2 \rightarrow 2H_2O + O_2 \rightarrow OFF$, respectively. The possibilities of sensor–target interactions can be simplified as follows: if sensor and target are involved in regulating one response, it is assumed that both are in a

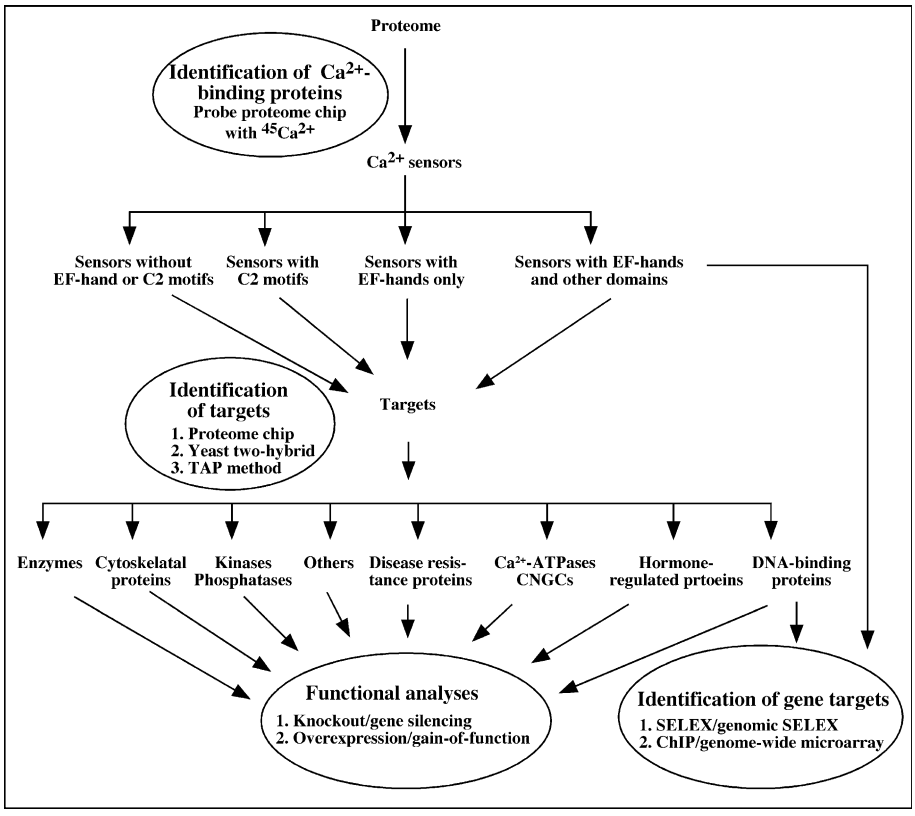

Fig. 5. Summary of proteomics and other approaches that can be used to identify Ca^{2+} signaling components, interactions among them and to analyze function of each component.

linear signaling circuit. Alternatively, if the sensor is involved in multiple responses and the target is involved in one of those responses mediated by the sensor, then it is assumed that the sensor has multiple targets and vice versa. The proteome chip sandwich assay (protein–protein-tag.AB) could be applied to study all possible interactions with an interested sensor or target (Fig. 2(b)). Further, fluorescence resonance energy transfer (FRET)-based analysis could be used to test in vivo interactions between sensors and targets for example between CPK and NPG or KIC and KCBP.

Antisense, knockout and overexpression analyses of genes encoding proteins with a C2 domain revealed their roles in abiotic and biotic stresses. Transgenic *Arabidopsis* expressing antisense AtPLC1 did not inhibit seed germination or growth in the presence of ABA, suggesting that PLC1 is necessary for ABA-mediated signaling (Sanchez and Chua, 2001). Silencing of PLDα in *Arabidopsis* retards ABA- and ethylene-promoted senescence whereas overexpression of bean PLDα in tobacco cells showed more rapid sensitivity to ABA. However, silencing PLDα in tomato cells showed reduced osmotic stress-induced PLD activation indicating PLDα mediates multiple processes (Fan et al., 1997; Sang et al., 2001). A Ca^{2+}-dependent cysteine proteinase activity has been reported in anoxia-induced roots of maize (Subbaiah et al., 2000) and in roots of *Arabidopsis* (Safadi et al., 1997). Recently, *DEK*1 (*defective kernel* 1), which encodes a calpain essential for grain aleurone development, has been cloned from maize using transposon tagging (Lid et al., 2002). DEK1 contains an N terminus C2 domain, 21 transmembrane domains and an intracellular cysteine proteinase domain. The proteolytic activity of DEK1 is enhanced in the presence of Ca^{2+} (Wang et al., 2003a). Two independent T-DNA insertion mutants with developmental defects led to isolation of a single copine gene, CPN1/BON1 (COPINE 1/BONZAI I). It contains two conserved C2 domains at the N terminus. The mutants show humidity and temperature-dependent developmental defects and accelerated hypersensitive response with constitutive expression of *PR* genes (Hua et al., 2001; Jambunathan et al., 2001). Overexpression of BAP1, a BON1-associated protein, restores the mutant phenotype (Hua et al., 2001).

5.4. Identification of gene targets of Ca^{2+} regulated DNA-binding proteins

Proteins with DNA binding domains with or without transcriptional activation domains have also been identified in Ca^{2+} signaling circuits (Tables 3 and 4). For

example, a Ca^{2+} sensor (At1g74430) and two small families of CBPs (members of EICBPs/SRs/CAMTAs and TGA.3) contain DNA-binding domains and may function as transcriptional activators for other genes. The specific *cis*-elements or target genes of DNA-binding proteins can be identified using systematic evolution of ligands by exponential enrichment (SELEX) or chromatin-immuno precipitation (ChIP) assays (Fig. 4). Screening of DNA arrays containing the whole genome with short genomic fragments (DNA) obtained in ChIP can also be used in a HT fashion to identify all of the target genes in the genome. Further, screening of cDNA microarrays with a cRNA probe prepared from a mutant that contains altered levels of DNA-binding protein as a result of loss-of-function or gain-of-function mutations would likely identify expression profiles of target genes in an HT manner (Fig. 4).

In SELEX assay, end-labeled random oligomers (random SELEX) or short genomic fragments (genomic SELEX) flanked by known primer sequences with appropriate restriction enzymes are incubated with a DNA-binding protein of interest and analyzed by an electromobility-shift assay (EMSA) in 5% non-denaturing polyacrylamide gels (Tuerk and Gold, 1990). Then, the DNA fractions from the resultant protein–DNA complex are isolated and amplified by PCR. The incubation, EMSA and PCR steps are repeated six to seven rounds and, finally, the identity of *cis*-elements that interact with the protein of interest is determined by sequencing DNA and searching the genome sequence (Fig. 4). Alternatively, a specific antibody can be used to identify its specific-targets in vivo using the ChIP assay (Fig. 4). The total in vivo protein:DNA complexes from a tissue of interest are cross-linked with formaldehyde fixation and used for chromatin isolation. Then the protein:DNA complex of interest can be pulled-down using a protein-specific antibody, a process called chromatin immunoprecipitation (ChIP) and purified by protein A-Sepharose. The recovered DNA can be separated from protein by heat, ligated to adapters, amplified by PCR and the protein binding sites are determined by sequencing and searching the database. Using this method, two targets of a MADS (AGL 15) protein have been identified from *Arabidopsis* embryonic cultures in which the AGL15 is highly expressed (Wang et al., 2002).

To locate the DNA-binding sequences in the genome, the immunoprecipitated DNA (IP-DNA) and its control non-immunoprecipitated DNA (NIP-DNA) can be labeled with Cy5 and Cy3, respectively, and used together to probe a genomic array containing genomic fragments. If IP-DNA binds more to a particular spot(s), then the DNA in that spot is a target of the protein used in the analysis (Fig. 4). This combined ChIP and microarray screening was successfully utilized to identify gene targets for two yeast transcriptional factors, Gal4 and Ste12 (Ren et al., 2000). In addition, screening of the cDNA microarrays (containing all expressed genes) with a cRNA probe from a mutant (loss-of-function or gain-of-function) and wild-type would yield information about altered gene expression profiles of possible target genes even if the interested DNA-binding protein is a member of a family (Fig. 4).

6. Conclusions and future perspectives

In plants, Ca^{2+} emerged as a versatile messenger over other small non-proteineous molecules in mediating a variety of developmental, hormonal and biotic/abiotic stress signals. To match the versatility of Ca^{2+} changes in response to signals (Table 1), there are about 700 (~3% of the *Arabidopsis* proteome) known protein components that function at various "nodes" of Ca^{2+} signaling (Tables 2–5) and the number is expected to increase as more and more genome-wide approaches are used. Plants have many unique Ca^{2+} signaling components and large families of plant-specific proteins at each "node". In a "mix and match" fashion, plants are thought to wire many circuits that form a "grid-like" pattern which control a variety of biological functions (Tables 3–6). Although we have identified many parts of Ca^{2+} signaling, many more are yet to be identified. Large scale high-throughput approaches should help fill this gap. The greatest challenge in this area is to understand the function of each component and how various components/parts of Ca^{2+} signaling interact to generate the "network".

Although it is clear that signals generate a distinct Ca^{2+} signature, how the signals regulate components involved in Ca^{2+} signatures (first node) is poorly understood. Currently, although 81 proteins with a transmembrane domain are present (Table 2) and some of their localization is known, their regulation by voltage, ligands, Ca^{2+}-CaM and proton-force (Fig. 1) and precise roles in generating a myriad of Ca^{2+} signatures in cytosol and subcellular compartments in response to signals remain to be discovered. Similarly, functions of the components at the second and the third "nodes"—the interactions among them and with other proteins—are not completely understood. The function of either sensor or target in many Ca^{2+}-mediated processes has been identified. In some cases, the function of sensor and target in a process is known but their regulation and interaction with other proteins have not been characterized.

Molecular genetic and gain- and/or loss-of-function analyses of Ca^{2+} signaling components have great potential in understanding the functions of individual components. Functional analysis of some components has led to their use in agricultural biotechnology. For example, involvement of CaMs in a broad spectrum of

disease resistance, the SOS3 pathway in salt tolerance, CPKs in cold, salt/drought tolerance, MLO mediated disease resistance against mildew in barley and roles of transporters in ion-balance has been shown (Table 6). Functional analyses of other Ca^{2+} signaling components would yield important insights that might be used in improving commercially important crops by introducing desirable traits. Because of the presence of families at each "node" and thereby their functional redundancy, it is difficult to unravel the roles of individual signaling components without prior knowledge of their possible regulators, downstream targets and functional protein complexes. Another main gap in Ca^{2+} signaling research is the circuit-specific cellular biochemistry that operates between targets and responses. To answer these questions, it is necessary to study Ca^{2+} signaling components at a genome level as summarized in Fig. 5. Screening of a proteome microarray with $^{45}Ca^{2+}$, a sensor or a target would provide a whole battery of sensors, targets and regulators of targets plus other interacting proteins, respectively (Fig. 2). Identification of the composition of functional protein complexes using affinity purification coupled with mass-spectrometry approaches (Fig. 3), target genes of transcriptional factors and their expression profiles with SELEX, ChIP cDNA/genomic microarrays (Fig. 4) and in vivo localization and protein–protein interaction studies using in situ hybridization and reporter gene fusion-based FRET analysis would provide valuable information in designing experiments for functional analysis of Ca^{2+} signaling components.

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