Chapter 4

Stress Signaling II: Calcium Sensing and Signaling

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Summary

Calcium is an essential second messenger in plant signaling networks. Many environmental and developmental stimuli induce an increase in cytosolic calcium to trigger different physiological responses. The specificity of Ca^{2+} signaling is achieved by a combination of distinct calcium signatures that are generated by specific calcium channels, pumps and transporters, and diverse calcium sensors that differ by their expression pattern, sub-cellular localization, substrate specificities and calcium sensitivities. Calcium binding modifies the structural conformation or enzymatic activity of the calcium sensors, which subsequently regulate downstream targets. Calmodulin is the most important Ca^{2+} transducer in eukaryotes and regulates numerous proteins with diverse cellular functions, including protein kinases. Plants also possess specific multigene families of protein kinases that play crucial roles in mediating calcium signaling. The multiplicity and diversity of plant calcium sensors, as well as the interconnections between various signal transduction pathways, constitute a tightly regulated signaling network that induces specific stress responses to improve plant survival.

Keywords Calcineurin B-like • calcium • calcium-dependent protein kinase • calcium sensing • calcium signatures • calmodulin • stress signaling

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I Introduction

Calcium is an essential plant nutrient that plays structural roles in the cell wall and membranes, and regulates plant growth and development (Hepler 2005). However, to avoid toxicity, calcium is maintained at low levels in the cytosol through the activation of calcium pumps and storage in multiple intracellular compartments as well as extracellular spaces (Fig. 1) (Sanders et al. 2002). While the role of calcium seems to be limited in prokaryotes (Dominguez 2004), it has evolved to be a ubiquitous second messenger in plants that mediates complex responses to developmental and environmental cues. Many external and internal signals can strongly, rapidly and transiently increase cytosolic calcium $[Ca^{2+}]_{cyt}$, through the regulation of diverse calcium transport systems (Fig. 1). The abundance of buffering calcium binding proteins in the cytosol can reduce calcium mobility and facilitate the localized and spatially distinct elevations in calcium concentrations (White and Broadley 2003). These calcium signals can be decoded by protein sensors which display an altered conformation and/or activity upon calcium binding. Understanding the specificity of calcium signaling has been a major challenge in plant biology for decades, since many diverse stimuli generate Ca^{2+} signals to trigger totally different responses. This signaling specificity can be achieved by different features of calcium signatures, distinct calcium sensitivities, expression and localization of calcium sensors and their downstream relay partners, as well as interactions with other signaling cascades. This review provides an overview of plant calcium signaling in response to abiotic stresses.

Abbreviations ABA – abscisic acid; ACA – auto-inhibited Ca^{2+}-ATPase; cADPR – cyclic ADP Ribose; CaM – calmodulin; CaMBP – calmodulin-binding protein; CAMTA – calmodulin-binding transcription activator; CBK – calmodulin-binding protein kinase; CBL – calcineurin B-like; CCaMK – calcium and calmodulin-dependent protein kinase; CDPK – calcium-dependent protein kinase; CIPK – CBL-interacting protein kinase; CML – calmodulin-like; CNGC – cyclic-nucleotide gated channel; cNMP – cyclic nucleotide monophosphate; CRK – CDPK-related protein kinase; DGK – diacylglycerol kinase; GABA – γ-aminobutyric acid; GAD – glutamate decarboxylase; IP_3 – inositol triphosphate; MAPK – mitogen-activated protein kinase; PA – phosphatidic acid; PI-PLC – phosphoinositide-specific phospholipase C; PLD – phospholipase D; SOS – salt-overly sensitive

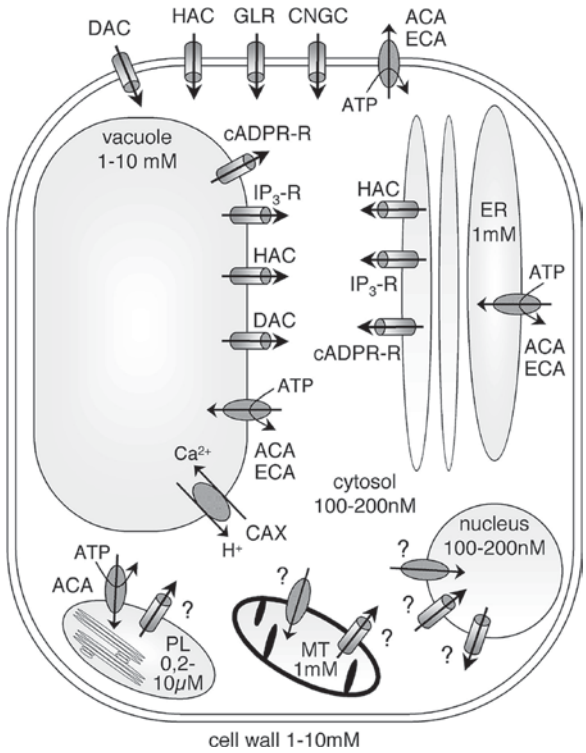

Fig. 1. Schematic representation of Ca^{2+}-permeable channels, pumps and transporters that are proposed to be involved in calcium signaling in response to abiotic stresses. Ca^{2+}-permeable channels (cylinders) can be regulated by voltage, either hyperpolarization (HAC) or depolarization (DAC) or ligands. The ligand-gated channels include IP_3 receptors (IP_3-R), cADPR receptors (cADPR-R), glutamate receptors (GLR) and cyclic nucleotide-gated channels (CNGC). Genes encoding HAC, DAC, IP_3-R and cADPR-R have not been identified in plants. Ca^{2+}-pumps and transporters (ovals) comprise ACA and ECA Ca^{2+}-ATPases, and the CAX Ca^{2+}/H^+-antiporters. Biochemical and electrophysiological evidence indicate the presence of Ca^{2+} transport systems involved in stress responses in the mitochondria (MT) and the nucleus, but their molecular identity is not clear yet. Currently, there is no evidence for the involvement of plastids (PL) in regulating abiotic stress Ca^{2+} signals. The estimated calcium concentration is indicated for each cellular compartment (Pauly et al. 2001; Reddy and Reddy 2004) (Adapted from Reddy and Reddy 2004).

II Calcium Signals

A *Calcium Signatures*

Valuable tools have been developed to monitor $[Ca^{2+}]_{cyt}$. Fluorescent dyes, like fluo-4, fura-2 and indo-1, allow single-cell calcium imaging, whereas the calcium-sensitive luminescent protein aequorin

can be expressed in different cellular compartments (Knight et al. 1991; Reddy and Reddy 2004). The cameleon probe, which is based on green fluorescent protein, has been adapted for plant systems to provide non-invasive features and high calcium sensitivity (Allen et al. 1999). Using these tools, increase in $[Ca^{2+}]_{cyt}$ has been monitored in response to many abiotic stresses in plants (Scrase-Field and Knight 2003; White and Broadley 2003). Calcium signals are defined by kinetic parameters (amplitude, duration, frequency, lag time) and spatial features (calcium origin and localization), and a particular combination of these factors appears to be specific to each stimulus (Table 1). The calcium response also depends on the strength of the stimulus, allowing a tight regulation of subsequent responses (Pauly et al. 2001). The use of calcium chelators or inhibitors of calcium channels indicates that different calcium sources are involved, depending on the stimuli (White and Broadley 2003). For example, similar calcium kinetics induced by cold and touch result from different calcium sources and locations (Knight et al. 1991; Wood et al. 2000), which eventually contributes to response specificity. Furthermore, refractory periods, during which seedlings can still respond to other stimuli, have been described (Price et al. 1994), further demonstrating that distinct signals mobilize calcium from different stores. In addition to the cytosol, abiotic stresses also induce calcium elevation in other cellular compartments, including the nucleus and mitochondria (Subbaiah et al. 1998; van der Luit et al. 1999; Pauly et al. 2001). Interestingly, the Ca^{2+} signatures of organelles are independent of the cytosolic Ca^{2+} signals (Pauly et al. 2000; Logan and Knight 2003). Calcium signatures are also cell type and organ-specific in response to various abiotic stresses (Kiegle et al. 2000; White and Broadley 2003).

B Role of Calcium Signatures

Because calcium changes have been associated with various downstream physiological responses to abiotic stresses (Reddy and Reddy 2004), calcium signatures may be relevant for encoding specific information for proper adaptation to distinct conditions. For example, impairing calcium signals with chelators or channel inhibitors reduces plant tolerance to freezing (Monroy et al. 1993) and heat shock (Gong et al. 1998), whereas calcium treatment increases plant survival. Although calcium has been proposed to act simply as a chemical switch (Scrase-Field and Knight 2003), several lines of evidence suggest that calcium signals can also carry specific information that distinguishes the various abiotic stresses. For example, in tobacco seedlings, wind and cold induce the expression of *NpCaM-1* in a Ca^{2+}-dependent manner. Although both stresses increase Ca^{2+} level in cytosol and nucleus, cytosolic calcium triggers *NpCaM-1* induction by cold, whereas nuclear calcium is responsible for *NpCaM-1* induction by wind (van der Luit et al. 1999). Thus, calcium elevation in the same cellular compartment may display different functions, depending on the stimulus. Recently, artificial cytosolic calcium transients have been shown to induce rapid transcriptome changes resembling abscisic acid (ABA) responses in *Arabidopsis* seedlings, further demonstrating that a particular calcium signal can induce specific gene expression patterns (Kaplan et al. 2006). Studies on stomatal regulation in guard cells also support a specific

Table. 1. Calcium signatures in response to abiotic stresses.

Stimulus	Features of the cytosolic calcium signal	Calcium stores
Cold shock	Rapid and transient Ca^{2+} peak (seconds)	Mainly external
Slow cooling	Bimodal Ca^{2+} elevation (minutes)	External and internal (vacuole, IP_3-dependent)
Hyperosmotic and salt stress	Single or biphasic Ca^{2+} elevation (20–60 s)	External and internal (vacuole, IP_3-dependent)
Hypoosmotic stress	Rapid and bimodal Ca^{2+} elevation (minutes)	External and internal (ER)
Mechanical stress	Rapid and transient Ca^{2+} peak (seconds)	Internal
Oxidative stress	Single Ca^{2+} peak (minutes)	External and internal
Anoxia	Rapid and sustained Ca^{2+} elevation (hours)	Internal (mitochondria)
Heat shock	Sustained Ca^{2+} increase (15–30 min)	External and internal

References: See review Scrase-Field and Knight (2003), White and Broadley (2003).

role of calcium signatures. In the *det3* mutant, the altered calcium signal, induced by oxidative stress, fails to trigger stomatal closure, while calcium responses to cold and ABA are maintained. Artificially imposing the calcium oscillations, observed in wild-type plants, restores stomatal closure in *det3*, indicating that the calcium signal itself carries the information that induces specific responses (Allen et al. 2000). In addition, pretreatment of seedlings with a stimulus modifies calcium signals induced by other stresses, suggesting that calcium may act as a memory signal to help adjust better to subsequent unfavorable conditions (White and Broadley 2003).

C Calcium Channels, Pumps and Transporters

Increase in $[Ca^{2+}]_{cyt}$ results from a combination of calcium influx into the cytosol via Ca^{2+}-permeable channels, according to the electrochemical potential, and calcium efflux out of the cytosol through energy-dependent calcium ATPases and transporters (Fig. 1) (Sanders et al. 2002). Ca^{2+}-permeable channels, which can be activated by hyper-polarization, depolarization or ligand binding, such as glutamate, inositol triphosphate (IP_3), cyclic ADP ribose (cADPR) and cyclic nucleotide monophosphate (cNMPs), have been found in many different plant membranes (White and Broadley 2003; Hetherington and Brownlee 2004). Although the molecular identity of these channels is mostly unknown, their activities in response to abiotic stresses and the ability of the ligands to elicit calcium signals have been well documented (White and Broadley 2003; Reddy and Reddy 2004; Peiter et al. 2005; Carpaneto et al. 2007). For example, IP_3 and cADPR can induce calcium release from the vacuole and trigger the induction of stress-responsive genes such as *RD29A* (Wu et al. 1997; Xiong et al. 2002). The recent annotation and cloning of genes encoding putative calcium channels provides important tools to study their involvement in generating calcium signals (Sanders et al. 2002). The glutamate receptor GLR3.3 mediates calcium entry into the cytosol (Qi et al. 2006) and over-expression of AtGluR2/GLR3.2 confers hypersensitivity to Na^+ and K^+ ions, but not to mannitol (Kim et al. 2001). Thus, AtGluR2/GLR3.2 may play a specific role in Ca^{2+}-mediated adaptation to ionic stresses. Recently, the Ca^{2+}-sensing receptor CAS has been shown to control the Ca^{2+}-resting level and to regulate IP_3 concentrations in *Arabidopsis* (Tang et al. 2007). Cyclic-nucleotide gated channels (CNGCs), that are activated by cNMPs, can conduct several types of cations, including calcium (Sanders et al. 2002; Lemtiri-Chlieh and Berkowitz 2004). However, the functional role of CAS and CNGCs in mediating abiotic stress signaling requires further investigation.

Calcium efflux from the cytosol allows replenishment of internal and external stores (Fig. 1), and a return to resting calcium levels, which may contribute to shaping the specific and distinct calcium signatures. Ca^{2+} pumps, whose expression is induced by salt stress, include the endoplasmic reticulum (ER)-type Ca^{2+}-ATPases (ECA or type IIA) and the auto-inhibited Ca^{2+}-ATPases (ACA or type IIB) (Fig. 1) (Geisler et al. 2000; Sze et al. 2000). Interestingly, the *Arabidopsis* vacuolar ACA4 restores growth on NaCl and mannitol in a mutant yeast strain, suggesting a positive role of ACA4 in plant stress tolerance (Geisler et al. 2000). Among the transporters, the vacuolar Ca^{2+}/H^+ antiporter CAX1, which is induced by cold, has been shown to negatively regulate the cold-acclimation response in *Arabidopsis* by repressing the expression of *CBF/DREB1* genes and their downstream targets (Hirschi 1999; Catala et al. 2003).

III Calcium Sensing and Signaling

Any modification in the concentration of calcium must subsequently be decoded in the targeted cells and organs to induce appropriate responses depending on the stimulus. Calcium sensors have been divided into two groups: the sensor relays, including calmodulin (CaMs) and calcineurin B-like (CBLs) proteins, and the sensor protein kinases, such as calcium-dependent protein kinases (CDPKs) as well as calcium and calmodulin-dependent protein kinases (CCaMKs). CaMs and CBLs do not possess any intrinsic activity and have to transmit the calcium-induced modification to target proteins, whereas CDPKs and CCaMKs are directly activated upon calcium binding (Fig. 2).

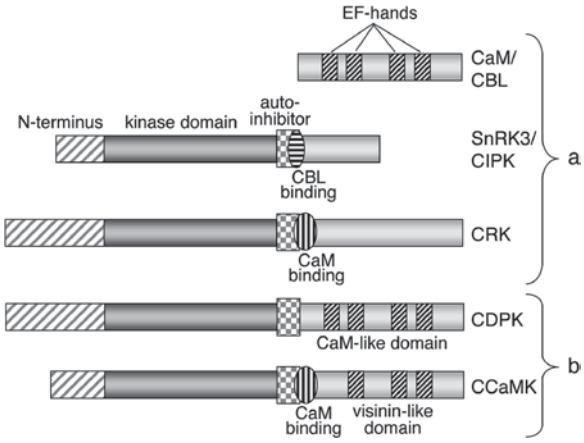

Fig. 2. Domain structure of plant calcium sensors. (**a**) Sensor relays and protein kinase partners. CaM and CBL are sensor relays that bind calcium through EF-hand motifs. CaM subsequently regulates many different target proteins including protein kinases (CRKs), whereas CBLs mainly activate CIPKs by interacting with the FISL/NAF domain (CBL binding) to release auto-inhibition; (**b**) Sensor protein kinases: In contrast to CRKs and CIPKs, the kinases CDPK and CCaMK can directly bind calcium through their EF-hand motifs. As a result, CDPKs function independently of other Ca^{2+} sensors whereas CCaMK activity can be further modulated by CaM (Adapted from Harper et al. 2004).

A Sensor Relays

1 Calmodulin and Calmodulin-Like Sensors

1.1 Biochemical Functions and Regulation of Calmodulin

Calmodulin is a small protein composed of two pairs of Ca^{2+} binding sites named EF-hands (Luan et al. 2002). Calcium binding modifies the CaM globular structure into an open conformation that allows interaction with proteins (Yamniuk and Vogel 2005). This interaction subsequently activates (Lee et al. 2000) or inhibits (Choi et al. 2005b; Yoo et al. 2005) CaM targets, translating a calcium signal into a biochemical response. The *Arabidopsis* genome contains seven *CaM* genes encoding four isoforms that differ by only one to four amino acids. In addition, *Arabidopsis* contains 50 genes encoding CaM-like (CMLs) proteins with more divergent sequences and sometimes extra-domains that confer additional properties (McCormack and Braam 2003). Specificity of CaM-mediated responses results from different expression patterns, specific targets, calcium affinities, sub-cellular localization and methylation (Luan et al. 2002; McCormack and Braam 2003). CaM isoforms differ in their ability to regulate target proteins (Lee et al., 2000; Yoo et al. 2005), possibly due to different structural interactions of the targets with CaM (Yamniuk and Vogel 2005). A recent protein array study has identified 173 protein targets of seven CaMs/CMLs in *Arabidopsis*. Among them, about 25% interact with all CaMs/CMLs tested, 50% with at least two of them, and 25% are specific to one CaM/CML (Popescu et al. 2007). CaMs sharing the same targets can compete for binding (Lee et al. 1999), indicating that target proteins are tightly regulated depending on the amount of each CaM isoform. Interestingly, a mutation converting three amino acids of rice OsCaM1 into those of OsCaM61, confers an ability to activate OsCBK almost as efficiently as OsCaM61 (Li et al. 2006). Thus, CaMs exhibit outstanding target specificities despite high levels of sequence identity. Different Ca^{2+} sensitivities were observed depending on CaM and target proteins, adding another layer of regulation (Lee et al. 2000; Luoni et al. 2006). CaMs also display multiple sub-cellular localizations (Yang and Poovaiah 2003). Interestingly, the petunia CaM53 and rice OsCaM61 are targeted to membranes or the nucleus depending on their prenylation status (Luan et al. 2002). Finally, CaM methylation may be a specific regulatory mechanism for a subset of target proteins (Roberts et al. 1986).

1.2 Calmodulin and Calmodulin-Like in Abiotic Stresses

The involvement of CaMs in abiotic stress responses was suggested by the reduced stress tolerance and gene expression observed after treatment with CaM antagonists (Monroy et al. 1993; Liu et al. 2003). In addition, expression of CaMs and CMLs is induced by touch, cold, heat shock or salinity (Luan et al. 2002; Yang and Poovaiah 2003). Also, it has been observed that heat shock enhances CaM protein level (Liu et al. 2003). Interestingly, over-expression of *Arabidopsis* CaM3 impairs cold induction of *RD29A*, *KIN1* and *KIN2* (Townley and Knight 2002), whereas *Arabidopsis* plants over-expressing the soybean GmCaM4 are more resistant to salinity (Yoo et al. 2005). This suggests a negative role of

CaM3 in cold signaling, while GmCaM4 positively regulates salt tolerance.

1.3 Calmodulin-Binding Proteins in Abiotic Stresses

As CaM has no enzymatic activity by itself, studying CaM-regulated proteins provides further evidence of CaM functions in abiotic stress responses. A large biochemical screen combined with computational analyses of homologs, identified about 100 putative CaM-binding protein genes *(CaMBPs)* in *Arabidopsis*. Most of these genes belong to multigene families, and some of them are induced by salinity, drought or cold (Reddy and Reddy 2004). In a recent protein array analysis, only a few newly identified CaM targets overlap with the previous study (Popescu et al. 2007), suggesting that the use of multiple strategies should facilitate the uncovering of the full spectrum of Ca^{2+}/CaM-regulated proteins. This discrepancy is probably due to the use of distinct expression libraries and methodologies. These results also indicate that differential regulation by CaM occurs among members of the same protein family. CaMBPs can be classified into two major groups: transduction proteins, such as protein kinases (CBKs) and transcription factors (CBTs) and effector proteins, including ion transporters and enzymes that directly function in physiological responses (Fig. 3).

Unlike in mammalian systems, CaM-regulated protein kinases (CaMKs) are not well characterized in plants. Apart from the chimeric CCaMKs (Fig. 2), only one protein kinase sharing similar structural features with mammalian CaMKs has been identified in apple (Harper et al. 2004). Plants also possess several CDPK-related protein kinases (CRKs) (Fig. 2), which are considered to be calcium-independent (Hrabak et al. 2003). However, new evidence suggests that some CRKs are stimulated by CaM in a Ca^{2+}-dependent manner (Harper et al. 2004). The specific up-regulation of *NtCBK2* by salt stress suggests that CRKs may function in salt tolerance (Hua et al. 2004).

It is intriguing that CaMs show both positive and negative effects on transcription factors.

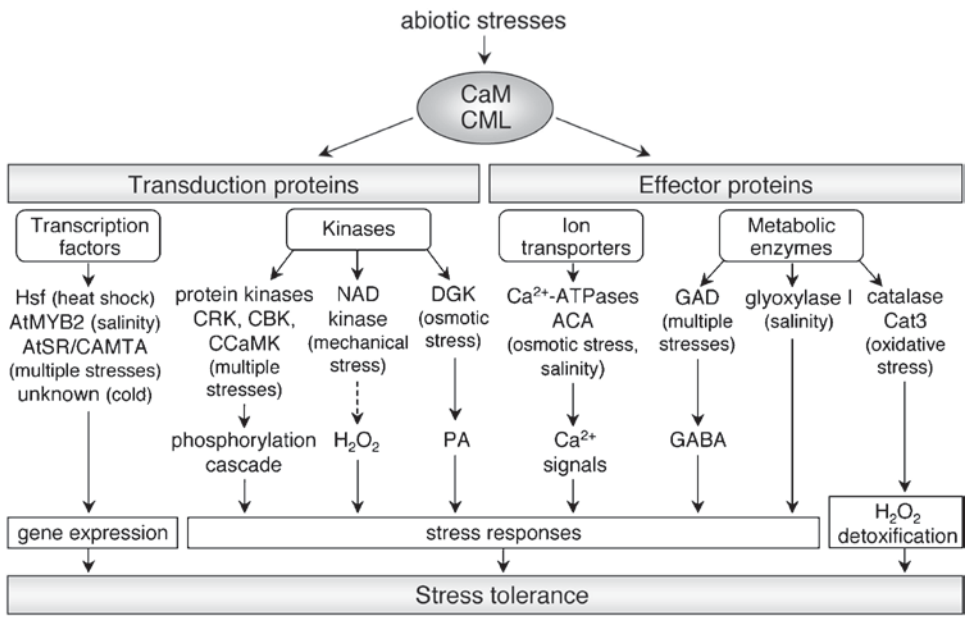

Fig. 3. CaM/CML functions in abiotic stress responses. The stress-activated CaMs/CMLs regulate multiple target proteins that are involved in diverse cellular processes such as transcription, signaling, ion transport and metabolism. Different types of kinases are responsible for initiating protein phosphorylation cascades, or inducing the direct (plain line) or indirect (dotted line) production of second messengers (H_2O_2, PA) to trigger stress responses. CaMs/CMLs also positively and negatively regulate transcription factors to modulate gene expression. Some specific roles, like H_2O_2 detoxification or generation of Ca^{2+} fluxes, have been established for several effector proteins regulated by CaM/CML. The underlying mechanism that leads to stress tolerance by modulating GAD and glyoxylase I is not clear. For each target protein, the activating stimuli are indicated in brackets.

During the heat shock response, CaM induces the expression of *HSP* genes (Liu et al. 2003) and increases the DNA binding of heat shock transcription factors (Li et al. 2004). GmCaM4 activates the transcription factor AtMYB2, and over-expression of GmCaM4 confers salt tolerance, that correlates with the enhanced expression of AtMYB2 target genes (Yoo et al. 2005). In contrast, CaM inhibits the transcriptional activation mediated by OsCBT (Choi et al. 2005b), which has similar structural features as *Arabidopsis* transcription activators AtSRs/CAMTAs (calmodulin binding transcription activator), which are induced by multiple stresses at the transcript level (Yang and Poovaiah 2003; Bouché et al. 2005).

Emerging evidence also suggests an involvement of Ca^{2+}/CaM in γ-aminobutyric acid (GABA) regulation, tolerance to oxidative stress, heat shock, as well as osmotic and salt tolerance through the regulation of effector proteins (Bouché et al. 2005). For example, glutamate decarboxylase (GAD), that triggers GABA accumulation in response to abiotic stresses, is activated by CaM in vitro (Lee et al. 2000). CaM has been proposed to play a dual role in regulating H_2O_2 homeostasis. On one hand, CaM induces H_2O_2 production by activating NAD kinase (Bouché et al. 2005). On the other hand, CaM induces detoxification by activating the catalase AtCat3 (Yang and Poovaiah 2002). Thus, CaM can regulate both effects of H_2O_2, i.e. mediate stress responses as a second messenger and induce cellular damage at higher concentration.

CaM is also a major regulator in salt and osmotic tolerance (Bouché et al. 2005). CaM stimulates the activity of glyoxylase I, an enzyme that positively functions in salt tolerance (Bouché et al. 2005). AtCaMBP25 is a small nuclear CaMBP, which plays a negative role in osmotic and salt tolerance (Perruc et al. 2004). In addition to regulation of protein activity, CaM also modifies cellular localization of target proteins. CaM recruits a tomato diacylglycerol kinase (LeDGK) to membranes where its substrate is located (Yang and Poovaiah 2003). As DGK produces phosphatidic acid (PA), involved in abiotic stress signaling (Xiong et al. 2002; Bargmann and Munnik 2006), CaM may play a positive role in stress responses by regulating PA signaling. Finally, CaM stimulates the activity of *Arabidopsis* type IIB Ca^{2+}-ATPases, ACA2 and ACA8, by releasing auto-inhibition (Hwang et al. 2000; Luoni et al. 2006). As ACA4 confers osmo-protection and resistance to salinity when over-expressed in yeast (Geisler et al. 2000), CaM may regulate calcium flux in response to multiple abiotic stresses.

2 Calcineurin B-Like Sensors

2.1 Structure and Functions of Calcineurin B-Like proteins in Abiotic Stresses

Like CaMs, CBLs are small proteins composed of two globular domains connected by a short linker. Each domain contains two EF-hand motifs harboring variable degrees of conservation compared to canonical sequences in CaMs, suggesting different Ca^{2+} capacities and affinities that most likely contribute to response specificity (Nagae et al. 2003). Crystal structure analysis has revealed that CBL2 binds two Ca^{2+} ions, while CBL4/SOS3 (salt-overly sensitive 3) binds four Ca^{2+} ions (Nagae et al. 2003; Sanchez-Barrena et al. 2005). Upon calcium binding, CBLs undergo conformational changes that allow hydrophobic interactions with other proteins (Sanchez-Barrena et al. 2005). In addition, several CBLs possess a putative myristoylation site that may promote membrane association (Kolukisaoglu et al. 2004). CBL4/SOS3 is myristoylated in vitro and associated with microsomal membranes (Ishitani et al. 2000), while CBL1 and CBL9 are targeted to the plasma membrane (Cheong et al. 2007). Interestingly, calcium binding also induces CBL4/SOS3 dimerization, which could reinforce membrane association (Sanchez-Barrena et al. 2005). Thus, the CBLs, comprising ten members in both *Arabidopsis* and rice (Kolukisaoglu et al. 2004), are calcium sensors that transmit the signal through protein interactions and can regulate the sub-cellular localization of their targets.

Exposure to cold, drought, salinity and ABA, differentially regulates *CBL* gene expression, suggesting a role for CBLs in abiotic stress responses (Batistic and Kudla 2004). SOS3/CBL4 was the first CBL identified by a genetic approach. The loss-of-function mutant is hypersensitive to salinity but displays the wild type response to osmotic stress. The mutant protein exhibits reduced calcium binding (Xiong et al. 2002). This indicates a specific role for SOS3/CBL4 in salt tolerance through calcium sensing. In addition, SOS3 myristoylation is required for salt tolerance, suggesting the importance of membrane association (Ishitani

et al. 2000). Recently, CBL10 has been shown to have overlapping functions with SOS3 in salt tolerance (Quan et al. 2007). Interestingly, CBL1 plays a broader role in regulating plant responses to salt, drought and cold (Albrecht et al. 2003; Cheong et al. 2003). The alteration of gene expression and the stress phenotypes of the mutant *cbl1* and *CBL1* over-expressing plants indicate that CBL1 is a positive regulator of drought and salt responses, but a negative regulator of the cold response (Cheong et al. 2003). While CBL1 exhibits ABA-independent functions, the closest related CBL9 acts as a negative regulator of ABA signaling, during germination and early development (Pandey et al. 2004). Surprisingly, unlike the *cbl1* and *cbl9* single mutants, the *cbl1cbl9* double mutant displays lower water loss under dehydration conditions due to ABA hypersensitivity for stomata closure (Cheong et al. 2007). Thus, CBLs exhibit complex redundant and specific functions, probably due to different expression patterns, interacting partners and cellular or sub-cellular localizations.

2.2 Calcineurin B-Like-Interacting Protein Kinases in Abiotic Stresses

CBLs share high sequence similarity to the regulatory subunit (CNB) of yeast calcineurin (CNA), a protein phosphatase involved in salt tolerance. However, yeast two-hybrid screens identified a family of Ser/Thr protein kinases (CIPKs) as the main plant CBL partner (Luan et al. 2002; Batistic and Kudla 2004; Reddy and Reddy 2004). CIPKs or PKS (SOS2-like protein kinases) belong to the SNF1-related protein kinase 3 (SnRK3) family, which possesses a unique C-terminal domain (Hrabak et al. 2003; Harper et al. 2004). The FISL/NAF domain in the C-terminus of CIPKs is sufficient for interaction with CBLs (Fig. 2), but the N-terminal domain contributes to the specificity of this interaction (Batistic and Kudla 2004). There are 25 and 30 CIPKs in *Arabidopsis* and rice, respectively, and differential CBL-CIPK interactions are detected even with closely related members (Batistic and Kudla 2004; Kolukisaoglu et al. 2004). Although these experiments were performed in the yeast two-hybrid system, they may reflect the formation of distinct CBL-CIPK complexes in plants. Accordingly, CBL-CIPK complexes exhibit different biochemical features in vitro, such as Ca^{2+}-dependence of interaction, cofactor and substrate specificity that may reflect different regulatory mechanisms in vivo resulting in response specificity (Luan et al. 2002; Batistic and Kudla 2004). CBL-CIPK interactions stimulate kinase activity and target the complex to plasma membrane, where CIPKs can phosphorylate specific substrates (Batistic and Kudla 2004; Gong et al. 2004; D'Angelo et al. 2006).

Differential stress induction of *CIPK* genes has been reported in distinct plant species, suggesting a role for these kinases in abiotic stress responses (Batistic and Kudla 2004). The most studied CIPK protein SOS2/CIPK24 was shown to be specifically involved in salt tolerance. Genetic analyses have demonstrated that the Na^+/H^+ antiporter SOS1, SOS2/CIPK24 and SOS3/CBL4 function in the same pathway (Xiong et al. 2002). SOS2/CIPK24 is inactivated by an intramolecular interaction, which is released upon binding to SOS3/CBL4 that senses salinity-induced calcium increase. Subsequently, SOS3/CBL4 targets the active kinase to the plasma membrane where it phosphorylates and activates SOS1, leading to Na^+ extrusion (Gong et al. 2004). Recently, CBL10 has also been shown to activate SOS2/CIPK24 and its downstream target SOS1 to trigger salt tolerance. Analysis of mutant phenotypes reveals that CBL10 mainly functions in shoot response to salt toxicity, whereas SOS3 primarily acts in roots (Quan et al. 2007).

The analysis of loss-of-function mutants indicates that CIPK3 is involved in cold and ABA-dependent salt stress responses, and positively regulates the early phase of stress-induced gene expression (Kim et al. 2003). CIPK1 mediates plant responses to osmotic stress, but not cold and salinity (D'Angelo et al. 2006). Interestingly, CIPK1 interacts with both CBL1 and CBL9, and the three loss-of-function single mutants exhibit hypersensitivity to osmotic stress. However, disruption of only CIPK1 or CBL9 impairs ABA responsiveness (Cheong et al. 2003; Pandey et al. 2004; D'Angelo et al. 2006). Thus, CIPK1 may regulate ABA-dependent and ABA-independent plant stress responses through alternative complexes with CBL9 and CBL1, respectively (D'Angelo et al. 2006). As freezing and salt tolerance are not affected in the *cipk1* mutant (D'Angelo et al. 2006), the functions of CBL1 in cold and salt signaling must be mediated by another CIPK. Considering the interactions detected in yeast two-hybrid assays, CBL1 may regulate CIPK24/SOS2 in response to salinity, but the partner in cold signaling remains to be iden-

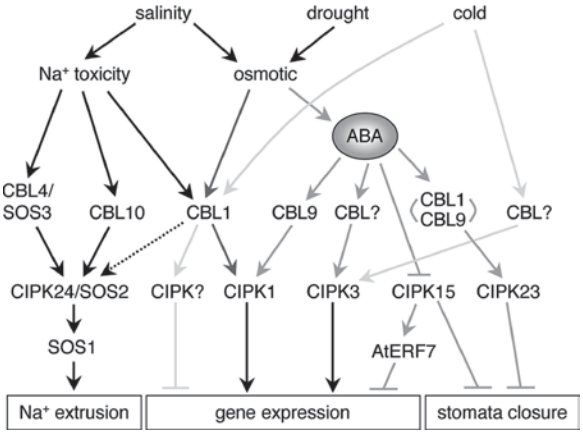

Fig. 4. CBL-CIPK signaling network in abiotic stress responses. Besides the well-studied SOS2/SOS3 pathway required for salt tolerance, other CBLs and CIPKs mediate regulation of gene expression and stomatal movements in ABA-dependent and ABA-independent pathways. While CBL4/SOS3 and CBL10 display overlapping functions to regulate CIPK24/SOS2 in different organs, CBL1 and CBL9 compete for CIPK1 regulation but act synergistically to modulate CIPK23. However, in some cases, only one partner has been shown to be involved in stress responses and specific CBL-CIPK complexes remain to be identified in vivo.

tified since CIPK3 does not interact with CBL1 (Kolukisaoglu et al. 2004). Recently, CBL1 and CBL9 were shown to act synergistically to activate CIPK23 and to inhibit ABA-dependent stomatal closure (Cheong et al. 2007). Furthermore, PKS3/CIPK15 was identified as a negative regulator of ABA signaling. In particular, the *pks3* mutant displays ABA hypersensitivity towards stomatal closure, leading to reduced water loss during dehydration (Guo et al. 2002). PKS3/CIPK15 also represses ABA-inducible genes through activation of AtERF7, a transcriptional repressor in ABA signaling (Song et al. 2005). These studies demonstrate that CIPK-CBL complexes form a highly regulated network through competition for partners, allowing a subtle regulation of calcium-dependent plant responses to abiotic stresses (Fig. 4).

B Sensor Protein Kinases

1 Calcium-Dependent Protein Kinases

1.1 Structure and Regulation of Calcium-Dependent Protein Kinases

CDPKs harbor a protein kinase domain linked to a CaM-like domain through a junction sequence that keeps the kinase inactive via a pseudosubstrate-binding mechanism (Fig. 2). The kinase activation results from intramolecular interaction between the CaM-like domain and the auto-inhibitory junction due to a Ca^{2+}-induced conformational change (Cheng et al. 2002; Harper et al. 2004). CDPKs are encoded by multigene families of 34 and 29 members in *Arabidopsis* and rice, respectively (Cheng et al. 2002; Asano et al. 2005). The significance of this multiplicity can be explained by the differences in Ca^{2+} activation thresholds, substrate recognition, expression patterns and sub-cellular localization (Cheng et al. 2002; Harper et al. 2004). It is likely that distinct CDPKs can sense and respond to different Ca^{2+} signatures. CDPKs display specificities on artificial substrates in vitro (Lee et al. 1998) that may reflect substrate specificities in vivo (Choi et al. 2005a; Rodriguez Milla et al. 2006).

Apart from Ca^{2+} activation, CDPK activity can be further modulated depending on isoforms. The identification of CDPK auto-phosphorylation sites in either the N-terminal variable domain, kinase domain or CaM-like domain suggests that they may differentially affect CDPK localization, activity, Ca^{2+} binding or protein interaction (Hegeman et al. 2006). Interestingly, 14-3-3 proteins that regulate enzymes after binding to phosphorylated sites can stimulate AtCPK1 activity (Camoni et al. 1998). Considering the variations in CDPK auto-phosphorylation sites, the 14-3-3 stimulation may represent a specific regulatory mechanism for a subset of CDPKs. CDPK activity is also modulated by phospholipids (Harper et al. 2004). Some of these phospholipids, like PA, act as second messengers (Xiong et al. 2002), which may play their signaling role through CDPK regulation. Generally, these phospholipids function as structural component of membrane and stimulate activity of CDPKs that are more active when associated with a membrane (Li et al. 1998). Importantly, CDPKs have been shown to localize in many different cellular compartments, including the nucleus, cytosol, chloroplast, peroxisome, ER and plasma membrane (Dammann et al. 2003; Harper et al. 2004). Myristoylation, an irreversible protein modification, is required for membrane targeting and insertion of CDPKs (Martin and Busconi 2000). Membrane association can be maintained by additional interactions, either via a cluster of positively charged amino

acids (Chehab et al. 2004) or by reversible palmitoylation (Martin and Busconi 2000). Thus, the unique structure of CDPKs provides an efficient co-targeting of a kinase and its Ca^{2+} regulator to coordinate Ca^{2+} sensing with cellular responses. It also allowed the co-evolution of kinases with divergent Ca^{2+}-binding domains to acquire the ability to respond to different Ca^{2+} signals.

1.2 Calcium-Dependent Protein Kinases in Abiotic Stress Signaling

Currently, only a few members of the CDPK protein family have been analyzed and shown to be specifically involved in stress responses. Progress has been slow because of the extensive functional redundancy of these proteins (Sheen 1996; Choi et al. 2005a). Expression of many CDPKs can be increased by abiotic stresses (Cheng et al. 2002). Transcriptional induction is consistent with the presence of stress-responsive *cis*-element in rice *CDPK* promoters (Wan et al. 2007) and correlates with enhanced protein levels (Abbasi et al. 2004; Yu et al. 2006). Furthermore, changes in intracellular localization of CDPKs have been observed in response to abiotic stresses. The groundnut AhCPK2 is translocated to the nucleus under hyper-osmotic conditions through an interaction with importins (Raichaudhuri et al. 2006). In the ice plant, McCPK1 moves from the plasma membrane to the nucleus after exposure to low humidity and salt stress (Patharkar and Cushman 2000; Chehab et al. 2004). Interestingly, the pseudo-response regulator transcription factor CSP1, which constitutes an in vitro substrate of McCPK1, is able to bind promoters of stress-inducible genes (Patharkar and Cushman 2000). In a maize protoplast transient expression assay, *Arabidopsis* CPK10 and CPK30, among several tested protein kinases, can specifically activate the promoter of the *HVA1* barley gene that is responsive to ABA, cold and salinity (Sheen 1996). Thus, CDPKs play positive roles in abiotic stress responses by inducing the expression of stress-responsive genes in both monocots and dicots.

Using a recombinant peptide substrate of CDPK (LCSP), an increase in a Ca^{2+}-dependent kinase activity was reported after oxidative stress in tobacco (Shao and Harmon 2003). Moreover, phosphorylation by a CDPK releases the feedback inhibition of an enzyme (serine acetyltransferase 2;1) involved in the biosynthesis of cysteine. Since the phosphorylation is induced by oxidative stress in vivo, CDPK may play a positive role in an anti-oxidative stress response by providing cysteine for glutathione production (Liu et al. 2006). CDPKs are also involved in cold signaling. In rice, a membrane-associated CDPK is activated after 18–24 h exposure to cold, suggesting a role in an adaptive process rather than in early responses (Martin and Busconi 2001). Similarly, OsCPK7/OsCDPK13 is activated by a 3 h cold treatment (Abbasi et al. 2004) and over-expression of either *OsCPK7/OsCDPK13* or *OsCPK13/OsCDPK7* confers cold tolerance in transgenic rice (Saijo et al. 2000; Abbasi et al. 2004).

Several lines of evidence indicate the involvement of CDPKs in drought responses. First, the dehydration-inducible gene *AtDi19* encodes a nuclear zinc finger protein that is a specific substrate of AtCPK4, 11 and 12 (Rodriguez Milla et al. 2006). Moreover, CDPK may reduce water loss under dehydration conditions by regulating diverse channel activities, such as the spinach aquaporin PM28A (Johansson et al. 1996). In faba bean guard cells, a CDPK phosphorylates the K^+ inward channel KAT1 in vitro (Li et al. 1998), which results in inhibition of the channel activity and contributes to stomatal closure (Berkowitz et al. 2000). In contrast, AtCPK1 activates a vacuolar Cl^- channel, resulting in Cl^- uptake into the vacuole and stomatal opening (Pei et al. 1996). Drought responses and stomatal movements are regulated by ABA. In the *Arabidopsis cpk3cpk6* double mutant, ABA-induced stomatal closure is reduced, concomitant with an impaired ABA activation of slow-type anion channels and calcium permeable channels (Mori et al. 2006). Thus, AtCPK3 and AtCPK6 are both positive regulators of stomatal ABA signaling. However, their functions may not be redundant since they belong to distinct CDPK subfamilies (Cheng et al. 2002). Significantly, ABA stimulates the activity of the grape berry ACPK1 (Yu et al. 2006), which positively regulates ABA-induced stomatal closure and the expression of stress-responsive genes (Yu et al. 2007). In *Arabidopsis*, AtCPK32 phosphorylates and activates the ABA-responsive transcription factor ABF4, leading to enhanced expression of ABF4 target genes (Choi et al. 2005a). It is likely that multiple CDPKs act through ABF4 and related transcription factors to activate ABA and stress signaling (Sheen 1996; Choi et al. 2005a).

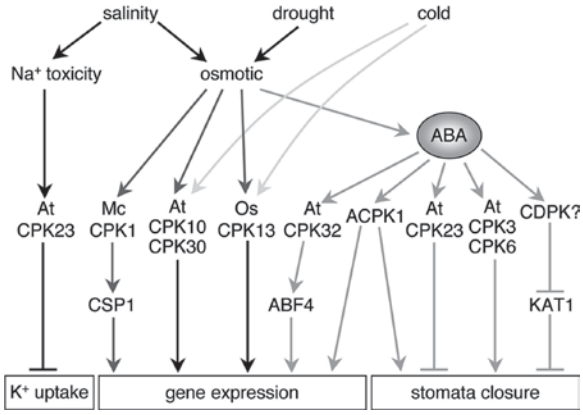

Fig. 5. CDPK signaling network in abiotic stress responses. CDPKs from different plant species regulate physiological responses to abiotic stresses, such as K^+ uptake, gene expression and stomatal aperture. While some transcription factors (CSP1, ABF4) and channels (KAT1) have been identified as CDPK substrates, most of the downstream components in CDPK signaling pathways are still unknown.

In rice, plants over-expressing *OsCPK13/OsCDPK7* exhibit enhanced resistance to drought and salinity, correlated with increased expression of stress-responsive genes (Saijo et al. 2000). A recent study has shown that AtCPK23, distinct from AtCPK10, 30 and 32 (Cheng et al. 2002), appears to have a negative function in drought and salt responses (Ma and Wu 2007). In addition, CDPK also regulates Ca^{2+} signatures by inhibiting the Ca^{2+} pump ACA2 located in the ER (Hwang et al. 2000). Thus, CDPKs mediate abiotic stress responses by regulating stomatal aperture, channel activities and gene expression (Fig. 5).

2 Calcium and Calmodulin-Dependent Protein Kinases

CCaMKs have been identified in different plant species, but there is no evidence for their presence in *Arabidopsis* (Harper et al. 2004). CCaMKs possess an N-terminal kinase domain followed by two regulatory domains: a CaM-binding domain which overlaps with an auto-inhibitory region and a visinin-like domain containing 3 EF-hands (Fig. 2) (Sathyanarayanan and Poovaiah 2004). This leads to a complex regulatory mechanism involving both Ca^{2+} and Ca^{2+}/CaM binding. In this model, the autophosphorylation induced by Ca^{2+} binding to the visinin-like domain increases CaM affinity, whose subsequent binding releases auto-inhibition and activates the kinase (Sathyanarayanan and Poovaiah 2004). Moreover, CCaMK activity is differentially modulated by CaM isoforms, adding another layer of regulation (Sathyanarayanan and Poovaiah 2004). In legume plants, CCaMKs play a critical role in Nod factor signaling and gene regulation essential for N_2 fixation (Gleason et al. 2006). So far, only one study reported the involvement of CCaMKs in stress responses. In pea roots, PsCCaMK localizes to the nucleus and its protein level increases after cold and salt stress (Pandey et al. 2002). The dephosphorylated form of the protein p40 binds to the promoter of the stress-induced *AtCaM5*, which may blocks *AtCaM5* expression. As PsCCaMK phosphorylates p40 in vitro, PsCCaMK may release the repression of *AtCaM5* under stress conditions (Pandey et al. 2002).

3 Other Calcium-Binding Proteins

Three different types of Ca^{2+}-regulatory motifs have been characterized: EF-hands, C2 domain and annexin fold. Bioinformatics analysis identified 250 EF-hand-containing proteins in *Arabidopsis*, including some that are known to be involved in abiotic stress responses (Reddy and Reddy 2004). Ca^{2+} binding has been confirmed for the bHLH transcription factor AtNIG1, which localizes to the nucleus, and specifically binds to E-box sequences that are present in the promoter region of many salt stress-inducible genes. Although the effect of Ca^{2+} binding is unknown, the *Arabidopsis* knockout mutant *atnig1-1* exhibits hypersensitivity to salinity stress, suggesting that AtNIG1 plays a positive role in salt tolerance (Kim and Kim 2006). Phosphoinositide-specific phospholipase C (PI-PLC) contains a C2 domain and an EF-hand motif, that is required for PLC activity (Otterhag et al. 2001). PI-PLCs are Ca^{2+}-dependent enzymes that trigger IP_3-dependent calcium release to modulate stress responses, including gene expression (Xiong et al. 2002; Reddy and Reddy 2004). As AtPLC2 is predominantly localized in the plasma membrane (Otterhag et al. 2001), PI-PLCs may sense early increases in cytosolic calcium and enhance the signal by inducing further calcium release.

Phospholipase D (PLD) α, β, γ, δ and ε, which require different calcium concentrations for activity, contain a C2 domain involved in Ca^{2+}-dependent phospholipid binding (Reddy and Reddy 2004).

PLDs are implicated in ABA signaling and stress tolerance, through the generation of PA, which acts as an important second messenger in plant stress responses or by inducing membrane remodeling (Bargmann and Munnik 2006). Interestingly, PLDα1 has been shown to mediate stomatal ABA signaling via a bifurcating pathway. On one hand, PA binding to ABI1 inhibits its phosphatase activity and leads to its sequestration to the plasma membrane, which then promotes stomatal closure. On the other hand, PLDα1 can also interact with the heterotrimeric G protein GPA1, while PA acts upstream of GPA1, leading to activation of the G protein and inhibition of stomatal opening (Mishra et al. 2006). The C2 domain is present in many other proteins whose biological function awaits future investigations (Reddy and Reddy 2004).

Annexins are small proteins that bind phospholipids in a Ca^{2+}-dependent manner (Sathyanarayanan and Poovaiah 2004). *Arabidopsis* genome contains eight annexin genes (*AnnAt*), that display differential induction by salinity, dehydration, cold and heat shock (Cantero et al. 2006). The protein levels of AnnAt1 and its association with the plasma membrane are increased by salt stress, and knockout mutants of AnnAt1 and AnnAt4 are hypersensitive to osmotic stress and ABA (Lee et al. 2004). This suggests that annexins may regulate target proteins at the plasma membrane to promote stress tolerance.

IV Conclusions

Calcium has emerged as an essential second messenger that mediates responses to developmental and stress stimuli in plants. Different signals have been proposed to elicit specific calcium signatures. Although several calcium channels and transporters have been identified at the molecular level, their specific roles in generating calcium signals in cytosol and sub-cellular compartments in response to stress remain to be elucidated. Understanding how these calcium signals are deciphered and relayed constitutes another challenge. Diverse plant calcium sensors are encoded by large multigene families, which provide robust redundant or unique functions to enhance plant's ability to adapt themselves to constantly changing environmental conditions. Response specificity is believed to occur through different calcium sensitivities, expression, cellular localizations and substrate regulation. It will be interesting to determine whether CDPKs and CIPKs have distinct or overlapping roles in stress signaling. In addition, cross-talk between Ca^{2+}-mediated transduction pathways contribute to highly modulated plant responses. For example, a subset of CDPK and CIPK proteins may also be regulated by CaM (Popescu et al. 2007), and AtCPK1 and CaM have opposite effects in regulating Ca^{2+}-ATPase activity of ACA2 (Hwang et al. 2000). Although some protein targets of calcium sensors have been identified, the molecular mechanisms underlying calcium signaling remain to be fully explored. As plant mitogen-activated protein kinase (MAPK) cascades are also key components in stress signaling, the interplays between calcium and MAPK signaling pathways require future investigation (details on some of these aspects have also been presented in Chapter 7 of this book). The interaction observed between the MAPK phosphatase NtMKP1 and a CaM suggests cross-talks between Ca^{2+}-dependent and Ca^{2+}-independent transduction pathways (Yamakawa et al. 2004). Thus, calcium and its sensors appear to be crucial nodes in the stress signaling networks that are essential in cross-tolerance, which increases plant survival under unfavorable conditions.

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