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FUNCTIONAL CHARACTERIZATION OF CALCIUM DEPENDENT PROTEIN KINASE 32 FROM ARABIDOPSIS

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FUNCTIONAL CHARACTERIZATION OF CALCIUM DEPENDENT PROTEIN KINASE 32 FROM ARABIDOPSIS

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ABSTRACT

Calcium-dependent protein kinases (CPKs) are major plant Ca^{2+} sensors, many of which have roles in plant stress responses. The Arabidopsis genome encodes 34 CPK isoforms. Here we report characterization of AtCPK32 gene function. Analysis of transgenic plants expressing pCPK32-GUS shows that CPK32 is highly expressed in roots, pollen and embryo, as well as leaf hydathodes, and the abscission zone of mature siliques. Real time RT-PCR and promoter expression patterns show that CPK32 is responsive to abiotic and biotic stresses. Plants treated with salt, ABA, osmotic stress (PEG), wounding, and flagellin 22 peptide show up-regulation of CPK32 upon these stress treatments. The overexpression of CPK32 results in ABA and salt insensitive phenotypes whereas disruption of CPK32 gene by T-DNA insertion leads to ABA and salt hypersensitive phenotypes in seed germination and early seedling growth assays. Interestingly, CPK32 overexpression plants are sensitive to drought whereas *cpk32-1* mutant plants are drought resistant suggesting that ABA and salt might be operating independent of drought stress tolerance. In a protoplast transient expression assay CPK32 is localized to the plasma membrane. Upon ABA treatment, CPK32 quickly moves from the plasma membrane to the cytosol and nucleus. Two key posttranslational modifications, myristoylation and palmitoylation play a crucial role in sub-cellular targeting of CPK32 to the plasma membrane. Mutation of these acylation sites leads to cytosolic and nuclear localization of CPK32 protein. Together our data provides evidence that CPK32 is a negative regulator of ABA signaling and is involved in multiple stress signaling pathways.

DEDICATION

I would like to dedicate this work to my mother, Mrs. Aparna Arun Gore for all her hard work and struggle to make me what I am today. Thank you for all your love, and constant support. This work would not have been possible without your encouragement. I love you and miss you very much.

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CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

Plants are sessile organisms and have to cope with a constantly changing environment. Their external conditions need to be evaluated in order for them to best utilize their resources and to respond to their growth conditions. There are two distinct types of external stimuli: biotic and abiotic. External biotic stimuli arise from direct interactions with pathogens and herbivores, as well as from indirect factors such as volatile compounds associated with herbivore attack and plant-microbe interactions (Baldwin et al., 2006). External abiotic stimuli include factors such as extremes of temperature or water, drought, flooding, osmotic stress, salinity, wind, and nutrient availability.

Plants use various types of messengers in order to coordinate their responses to external environmental signals. These external signals are decoded to produce downstream effects, generally using different membrane receptors, non-protein messengers, metabolite transporters, protein kinases and phosphatases, transcription factors, and early response genes. While use of particular receptors, protein effectors, transcription factors, and promoters specify a given signaling pathway, how response specificity is achieved through a limited number of second messengers is generally not clear. When compared to the number of proteins involved in signal transduction pathways, there are fewer second messengers utilized by cells. These include Ca^{2+},

cyclic nucleotides (cAMP, cGMP), reactive oxygen species, H^+, and a variety of lipids such as diacylglycerol and phosphoinositides (Sanders et al., 2002; and references therein). Among second messengers, Ca^{2+} has an unusual role in both plants and animals since it also has direct metabolic functions as well as signaling functions. The functions of Ca^{2+} in plant cells will be discussed in the following paragraphs.

Physiological Functions of Calcium in Plants

Calcium is a crucial micro-nutrient required for the growth and development of plants. Ca^{2+} acts as a counter cation for inorganic and organic anions in the vacuole (White and Broadley, 2003). Ca^{2+} deficiency generally does not affect plant growth as severely as does deficiency of other macro-nutrients or even many micro-nutrients. Therefore, Ca^{2+} generally has less impact on agriculture (Hepler, 2005). However, low Ca^{2+} can result in organ specific defects such as poor root development, leaf necrosis and curling, fruit cracking, blossom end rot, and poor shelf life of storage produce (White and Broadly, 2003). However, even though Ca^{2+} is critical for plant growth, excessively high levels of Ca^{2+} are detrimental to plants, leading to necrosis, nutritional imbalance, and changes in pH of the soil. This is a particular problem for growth on calcareous soils (White and Broadly, 2003).

At the cellular level, Ca^{2+} is important for maintaining structural integrity of the cell wall and cellular membranes, and is required for membrane permeability. For example, during cell wall biogenesis Ca^{2+} binds to acidic pectin residues, forming Ca^{2+}-

pectate. This improves the structural integrity of the plasma membrane by binding Ca^{2+}-pectate to phospholipids, thereby helping to stabilize lipid bilayers. A Ca^{2+} concentration between 0.1 to 1 mM is necessary to preserve membrane integrity and selective ion transport through the plasma membrane. Ca^{2+} is required at similar levels in media used for isolating plant protoplasts (Yoo et al., 2007). Ca^{2+} presence is necessary also for potassium transport in the presence of sodium, by allowing the selectivity for ion transport (Epstein et al., 1961).

Ca^{2+} is responsible for polarized cell growth during pollen germination, pollen tube growth and elongation, as well as root hair elongation. Ca^{2+} is asymmetrically distributed in the growing pollen tube, with its highest concentration in the tip. The Ca^{2+} concentration gradient provides polarity to the elongating cells (Rathore et al., 1991; Miller, 1992). Along with these functions, cytosolic Ca^{2+} concentration has a major role in the depolymerization of microtubules during cell division, in cytoplasmic streaming in internode cells of algae, and in the regulation of gas exchange through leaf stomata (McAinsh et al., 1997).

Calcium Functions as a Second Messenger

Apart from being nutritionally important, Ca^{2+} acts as a second messenger in a variety of signal transduction pathways in plants. Within plant cells, Ca^{2+} is stored in several vesicular compartments. Through the activities of membrane channels and ion pumps, Ca^{2+} is able to move into and out of cells as well as cellular storage

compartments. The extracellular space (apoplast), vacuole, and endoplasmic reticulum (ER) are able to store high levels of Ca^{2+}, as compared to mitochondria and chloroplasts. Ca^{2+} concentrations in ER and vacuole can become as high as 50 mM (Bush, 1995), but the cytoplasmic concentration is maintained at very low levels which helps to prevent Ca^{2+} cytotoxicity (Tuteja and Sopory, 2008). Normally, under resting conditions, the free cytosolic [Ca^{2+}] concentration ranges between 30-200 nM (Bush, 1993) even though the total Ca^{2+} concentration is higher due to Ca^{2+} that is bound to a number of Ca^{2+} binding proteins (Tuteja and Sopory, 2008).

In response to different abiotic and biotic stimuli, the cytosolic Ca^{2+} concentration in a cell can change rapidly. This change in Ca^{2+} concentration is transient and leads to an array of spatial and temporal patterns of Ca^{2+} accumulation within cells. These stimulus-specific spatiotemporal patterns in Ca^{2+} concentrations are called "Ca signatures" (McAinsh, 1992). Ca signatures are generated by the flow of Ca^{2+} through various Ca^{2+} channels associated with calcium stores with high electrochemical potential (Ng and McAinsh, 2003), followed by the removal of Ca^{2+} from the cytosol by different pumps. Such patterns of rapid changes in Ca^{2+} concentrations lead to Ca^{2+} oscillations, or waves, which vary in amplitude, duration and frequency (Fig. 1.1). These patterns give specificity to each Ca signature (McAinsh et al., 1995).

Ca signatures vary according to the nature and strength of stimuli such as osmotic stress, salt and drought stress, oxidative stress, gaseous pollutants, temperature changes, light, type of pathogen elicitors, plant hormones, bacterial and fungal signals, as well as mechanical and gravitational stimulations (Fig. 1.1; Knight and Knight, 2001; McAinsh

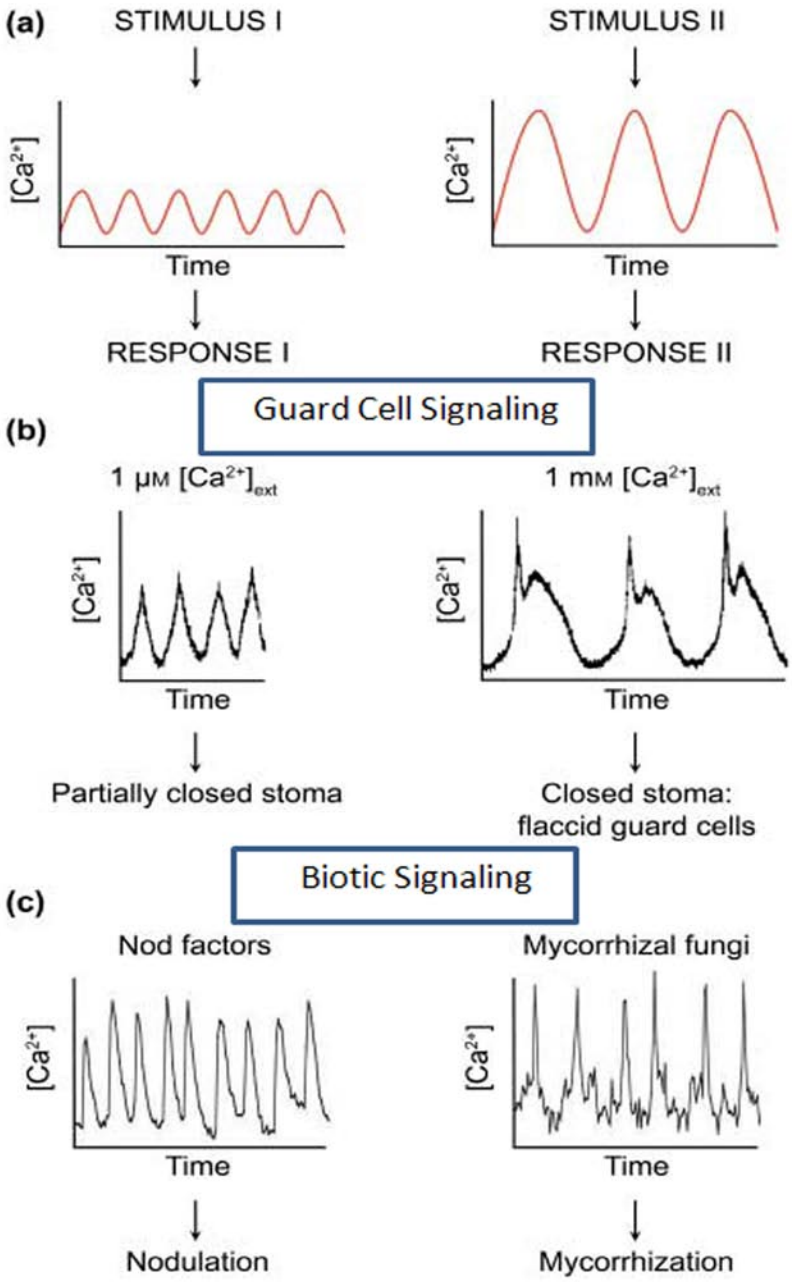

Figure 1.1. Different patterns of Ca signatures. (a) Schematic representation of temporal dynamics of Ca^{2+} oscillations. (b) Ca signatures produced in response to externally applied Ca^{2+} in *Commelina* guard cells in concentration dependent manner. (c) Ca signatures produced in response to rhizobial nod factor and mycorrhizal fungi in *Medicago* root hairs (modified from McAinsh and Pittman, 2009).

and Pittman, 2009; Qudeimat and Frank, 2009). The pattern of Ca signatures produced in response to a single stimulus also depends upon the plant developmental stage and the tissue or cell type. The specificity of Ca signatures and plant responses depend on several factors. Sometimes similar Ca signatures produce different end responses, perhaps as influenced by their cellular context. Other times, different stimuli can lead to different Ca signatures, but produce similar end responses. For example, cold stress produces biphasic Ca signatures throughout all the cells in a plant, but also leads to monophasic Ca signatures only in root cells (Knight et al., 1996; Knight et al., 2000). Both types of Ca signatures activate common transcription factors to generate downstream responses. On the other hand, osmotic stress and salt stress produce similar spatiotemporal patterns of Ca signatures, but involve different downstream components which produce a response to the stress. Salt stress leads to activation of salt overly sensitive (SOS) genes, but osmotic stress does not (Scrase-Field and Knight, 2003). The release of Ca^{2+} into the cytosol largely depends upon the specific stimulus, which in turn, influences a specific Ca^{2+} store to release Ca^{2+} into the cytosol. For example, Ca^{2+} is directly taken up from the apoplastic space in response to pathogenic elicitors, the phytohormone gibberelic acid, and water stress. However, in response to cold stress, Ca^{2+} is released to the cytosol from extracellular as well as intracellular stores, through the actions of inositol-3-phosphate (IP3) intermediates. The increase of cytosolic Ca^{2+} in response to wind or touch is carried out by the release of Ca^{2+} only from intracellular Ca^{2+} stores. Thus, specific stimuli influence cytosolic Ca^{2+} concentration by regulating different Ca^{2+} stores and their associated mechanism of Ca^{2+} release. The amplitude of

the Ca signature also corresponds to the type or severity of a stimulus. The cytosolic Ca^{2+} concentration is returned to pre-stimulus levels by efflux of Ca^{2+} from the cytosol to the cellular Ca^{2+} stores. This efflux is carried out by Ca^{2+} pumps, Ca^{2+} carriers or Ca^{2+} efflux transporter, such as high affinity Ca^{2+}-ATPase and low affinity Ca^{2+}/ H^{+} antiporters. Ca^{2+}-ATPases belong to the P-type ATPases which use ATP to carry out Ca^{2+} transport. On the other hand, the Ca^{2+}/ H^{+} antiporters use a pH gradient generated by vacuolar proton pumps to carry out Ca^{2+} transport (McAinsh and Pittman, 2009). Ca^{2+}-ATPases are either the ER type (ECAs) or the autoinhibited ATPase type (ACAs). Both ECA and ACAs are associated with ER, plasma membrane and tonoplast. Plant ECAs are transcriptionally regulated by several stresses and hence are associated with stress signaling (Maathuis et al., 2003). ACAs are involved in Ca signaling and are quickly activated by different calmodulins (McAinsh and Pittman, 2009). Thus, a transient change in cytosolic Ca^{2+} concentration by cellular influx and efflux of Ca^{2+} ions is due to Ca signatures interpreted by Ca^{2+} sensors, or decoders, which produce downstream responses.

In plants, there are three types of calcium decoders, the calmodulins (CaM, Yang and Poovaiah, 2005), the calcineurin β-like proteins (CBL; Luan et al., 2002) and the calcium dependent protein kinases (CPK; Harper et al., 2005). All three Ca^{2+} sensors possess helix-loop-helix structures called EF hands, which bind Ca^{2+}. Binding of Ca^{2+} to the sensors result in a conformational change, which in turn influences the interactions of these sensors with a variety of target proteins. CPKs are unique in their function since

they are both sensor relays and sensor responders. They undergo a conformational change upon Ca^{2+} binding and then further transduce the signal downstream by phosphorylating different effecter proteins and enzymes. CaM and CBL, on the other hand, are called sensor relays because they lack a responder domain that can modify activity of other proteins and enzymes. Apart from these Ca^{2+} sensors, there are other Ca^{2+} binding proteins which do not have EF hands, but can be involved in Ca^{2+} signaling. These include phospholipase D, annexins, calreticulin, and calnexins among others (Tuteja and Sopory, 2008). The characteristics and functions of the three major Ca^{2+} sensors, are discussed below.

OVERVIEW OF CALCIUM SENSORS IN PLANTS

Calmodulin

CaM is a small acidic protein and has no enzyme activity. CaM is highly conserved in all eukaryotes, having about 70% amino acid sequence identity between plants and animals (Yang and Poovaiah, 2003). CaMs are present as a multigene family in a given species. In plants, as well as in animals, CaMs have four EF hands implanted in two separate globular regions in the N-terminus and the C-terminus. These are separated by a flexible central helix called the linker domain, which leads to formation of a dumbbell shaped structure (Natalie et al., 1989). Upon Ca^{2+} binding to the EF hand, CaM undergoes a conformational change which exposes the hydrophobic core and allows

its binding to other proteins to regulate their activity (Hoeflich and Ikura, 2002). Thus, changes in cytosolic Ca^{2+} concentration can activate CaM for signal transduction by altering the activity of CaM-bound proteins.

In Arabidopsis there are seven CaMs and about fifty CaM-like proteins (CML; McCormack and Braam, 2003). CML proteins are considered as isoforms of CaM if they have four EF hands, have about 148 amino acids, and have at least a 50% identity to vertebrate CaM (Yang and Poovaiah, 2005). Unlike CaM, which has four EF hands, the number of EF hands in CML proteins ranges between three and six. The EF hand sequences are highly conserved among the CMLs, whereas the loop sequences show divergence from the CaMs (McCormack et al., 2005).

Most CaMs are expressed in the cytosol, except for a few that are expressed in peroxisomes, the nucleus, or the extracellular matrix (Yang and Poovaiah, 2003). At the tissue level, all Arabidopsis CaMs are expressed in almost every plant organ and during all developmental stages. In contrast, CMLs have more specific expression patterns. For example, some CMLs are highly expressed in floral organs such as stamens, whereas other CMLs are specific to leaves, cotyledons and sepals (McCormack et al., 2005). External stimuli have little effect on changes in the expression patterns of CaMs, but they do affect the expression of CMLs by several-fold. Expression of CMLs also depends on type and strength of the stimulus, maintaining a relationship between a specific CML and the stimulus. Expression of some CMLs is affected by one or more stimuli such as pathogen attack, wounding, and salt treatment. For example, microarray analysis of

Arabidopsis shows that expression levels of CML37 and CML39 were increased by 100 and 60-fold, respectively, by treatment with salt (McCormack et al., 2005).

Advances in protein-protein interaction screens have identified several CaM binding proteins. Even though CaM does not have any enzyme activity, the Ca^{2+} bound forms of CaM interact with diverse proteins, which include metabolic enzymes, protein kinases, phosphatases, cytoskeleton associated proteins, ion transporters, and transcription factors. Several studies have shown that CaM binding removes the autoinhibition of many target proteins such as Ca^{2+} ATPases, CaM-dependent kinases, and glutamate decarboxylase (Bouche et al., 2005). Arabidopsis CaM7 has been reported to interact with two Z box binding transcription factors, ZBF1/MYC2 and ZBF2/GBF1, to regulate expression of light inducible genes such as *RBCS1A* and *CAB1* (Yadav et al., 2005; Mallappa et al., 2006).

Calcineurin β-Like Proteins

CBL proteins also are highly conserved in all eukaryotes, from yeast to mammals. Calcineurin is a heterodimer containing a catalytic subunit, calcineurin A, and an associated regulatory subunit calcineurin β for calcium binding (Rushnak and Mertz, 2000). Calcineurin A is a protein phosphatase which possesses a CaM binding domain with EF hands and is attached to the calcineurin β subunit, which is required for activation of the phosphatase by the linker region. Though plants lack calcineurin, they do have homologs of the calcineurin β subunit, which are designated calcineurin β-like

proteins. These have about 32% identity to rat calcineurin β (Rushnak and Mertz, 2000). Each CBL protein contains four EF hands, of which the first shows amino acid substitutions of a critical Thr for Ca^{2+} binding site in activation loop renders the CBL inactive (Batistic and Kudla, 2004). Due to their conserved number and spacing of EF hands, CBLs have a highly conserved 3D structure (Kolukisaoglu et al., 2004).

CBL genes have similar structural domains, but differ in the length of their coding sequences. A genetic screen identified the salt overly sensitive 3 (sos3) mutant, which has about 30% identity with calcineurin β and with a neuronal Ca sensor from other organisms (Liu and Zhu, 1998; Kudla et al., 1999). The SOS3 protein is now designated CBL4. Plant CBLs also differ from animal calcineurin in their downstream targets. Unlike animal and fungal calcineurin β, which interact with their linked phosphatases, plant CBLs interact with protein kinases, designated as CBL interacting protein kinases (CIPKs). These kinases belong to the family of sucrose non-fermenting kinase (SNF1), referred to as the SNF related kinase group 3 (SnRK3; Shi et al., 1999; Hrabak et al., 2003).

Most CBL proteins are localized to the plasma membrane or the vacuolar membrane. The plasma membrane localized CBLs require post translational modifications such as myristoylation and palmitoylation for proper membrane targeting (Batistic and Kudla, 2009; and references there in). CIPKs on the other hand do not have plasma membrane localization signals and have both cytoplasmic and nucleoplasmic localizations. However, the CBL/CIPKs complexes are targeted to various membrane bound compartments by their respective interacting CBL proteins

(Batistic and Kudla, 2009). The number of CIPKs that an individual CBL can interact with shows a general evolutionary trend in the plant kingdom. In algae, there is a single CBL protein and a single CIPK. In moss, there are 4 CBL proteins and 7 CIPKs. In higher plants such as Arabidopsis, rice and poplar, there are 10 CBLs and 26 to 30 CIPKs (Batistic and Kudla, 2009). This suggests co-evolution has occurred between CBLs and CIPKs to generate functional diversity and output specificity of a given CBP/CIPK complex. A particular CBL can interact with one or more CIPKs, forming a stimulus-specific CBL/CIPK complex (Batistic and Kudla, 2009).

Several CBL/CIPK complexes have been assigned physiological functions. CBL/CIPK complexes often regulate the function of membrane channels and transporters (Hedrich and Kudla, 2006). For example, the Arabidopsis CBL4/CIPK24 complex is involved in regulating sodium toxicity (Halfter et al., 2000; Qui et al., 2002). Even though CBLs are represented by a multigene family, they maintain functional specificity by interacting with specific CIPKs and by forming many functional pairs. In Arabidopsis, CBL1 and CBL9 are two highly related CBLs that both interact with CIPK1, but they form alternative complexes which lead to functional diversity for osmotic stress regulation. CBL9 also interacts with CIPK3 to regulate ABA responses in seed germination. Both CBL1 and CBL9 interact with CIPK23 to regulate K^+ uptake and stomatal movements (Chinnusamy et al., 2004). Also, during salt stress, SOS3/CBL4 interacts with CIPK24/SOS2 and regulates SOS1, a plasma membrane located Na^{2+}/H^+ anitporter. This leads to detoxification by removing excess cytosolic Na^+ from cells in roots (Kim et al., 2007).

CPK-SnRK Superfamily

In Arabidopsis there are over 1000 protein kinases, of which at least 67 have been associated with Ca^{2+} signaling (Harper et al., 2004). These all belong to the CPK-SnRK superfamily (Hrabak et al., 2003). The CPK-SnRK superfamily consists of five types of kinases based on their structures and sequence similarities, as shown in Fig. 1.2. But, Sometimes phosphoenolpyruvate carboxylase kinase (PPCKs) and PEP carboxylases kinase-related kinases (PEPRK) are also included in CPK-SnRK superfamily (Hrabak et al., 2003). First, the CPK gene family has both a C-terminal CaM-like domain for Ca^{2+} binding and an N-terminal kinase domain with regulatory functions. Second, the CPK related kinase (CRKs) gene family shows a high similarity to the regulatory domain of CPK, but differs in the CaM-like domain. The CaM-like domain of CRK has degenerate EF hands and it is not clear if these proteins can bind Ca^{2+}. There are reports that CRKs can bind to CaM and hence, CRKs are sometimes also referred to as CaM-binding kinases (Wang et al., 2004 ; Leclercq et al., 2005). Third, the Ca/Calmodulin regulated kinases (CCaMKs) bind to both Ca^{2+} and CaM. CCaMKs are similar to CPKs, but have three EF hands, and therefore more closely resemble the brain visinin protein than does CaM (Patil et al., 1995). CCaMKs bind to Ca^{2+} as well as CaM. Other Ca regulated kinases include SnRKs (CIPKs) and calmodulin dependent protein kinases (CaMK), both mentioned earlier.

CALCIUM DEPENDENT PROTEIN KINASES

Multigene Family of CPKs

Hetherington and Trewavas (1982) were the first authors to identify CPK activity in plants, from pea (*Pisum sativum*) shoot membranes. The first CPK was isolated from soybean (*Glycine max*) by Harmon et al. (1987). CPKs have since been found across the plant kingdom, from algae to higher plants. CPKs are Ca^{2+} sensors found predominantly in plants and some protozoa, but absent in other organisms such as bacteria, fungi, yeast, flies, and animals (Cheng et al., 2002).

CPKs are represented by a multigene family in a given plant species. In Arabidopsis, there are 34 CPK genes (The Arabidopsis Genome Initiative, 2000), whereas in rice there are 31 CPK genes (Ray et al., 2007). Recent advances in genome sequencing projects have revealed that other species such as soybean, tomato, grapes, and maize have multiple CPK genes. For example, a recent study by Li et al. (2008) identified 20 CPK genes in wheat.

Arabidopsis CPKs are divided into four lineage groups (I-IV) based on their overall sequence similarities (56% to 96%). These are distributed over all five chromosomes (Cheng et al., 2002). Isoforms belonging to the same or even different subgroups can show distinct or overlapping functions. For example, AtCPK3 and AtCPK6 belong to subgroups II and I, respectively. These show a similar intracellular localization pattern, and both function in ABA related stress signaling and in stomatal movements (Mori et al., 2006). These factors, along with the genome wide sequence analysis and chromosomal distributions, suggest that gene duplications and divergence might have led to redundant, as well as distinct functions of given CPKs (Cheng et al.,

2002). Li et al. (2008) have compared several CPKs from wheat with ones from Arabidopsis, tomato and barley for possible correlations between the involvement of CPKs in stress responses and their sequence homology. This phylogenetic analysis showed a close sequence relationship between CPKs that are involved in similar functions in these species. Though wheat CPKs share functional and sequence similarity with other CPKs, further phylogenetic analysis involving a more diverse group of plants is needed to establish possible relationships between sequence homology and the functional similarity or redundancy of CPKs in general.

Domain Structure

Individual CPK proteins have four domains, an N-terminal domain, a serine-threonine protein kinase domain, an autoinhibitory domain, and a CaM-like domain (Fig. 1.2). Having the CaM-like domain allows direct activation of these proteins by Ca^{2+} binding. CPKs function as monomeric proteins and can potentially interact with many other proteins (Cheng et al., 2002). The amino acid sequences of the N-terminal variable domain (NTV) have a lot of sequence divergence and ranges from 40 to180 amino acids in length (Harmon, 2003). Even though this is a highly variable domain, it conserves crucial information for sub- cellular localization and possible membrane targeting (Cheng et al., 2002; and references therein). Traditionally, CPKs were not predicted to be membrane proteins. However, most CPKs possess two post translational lipid

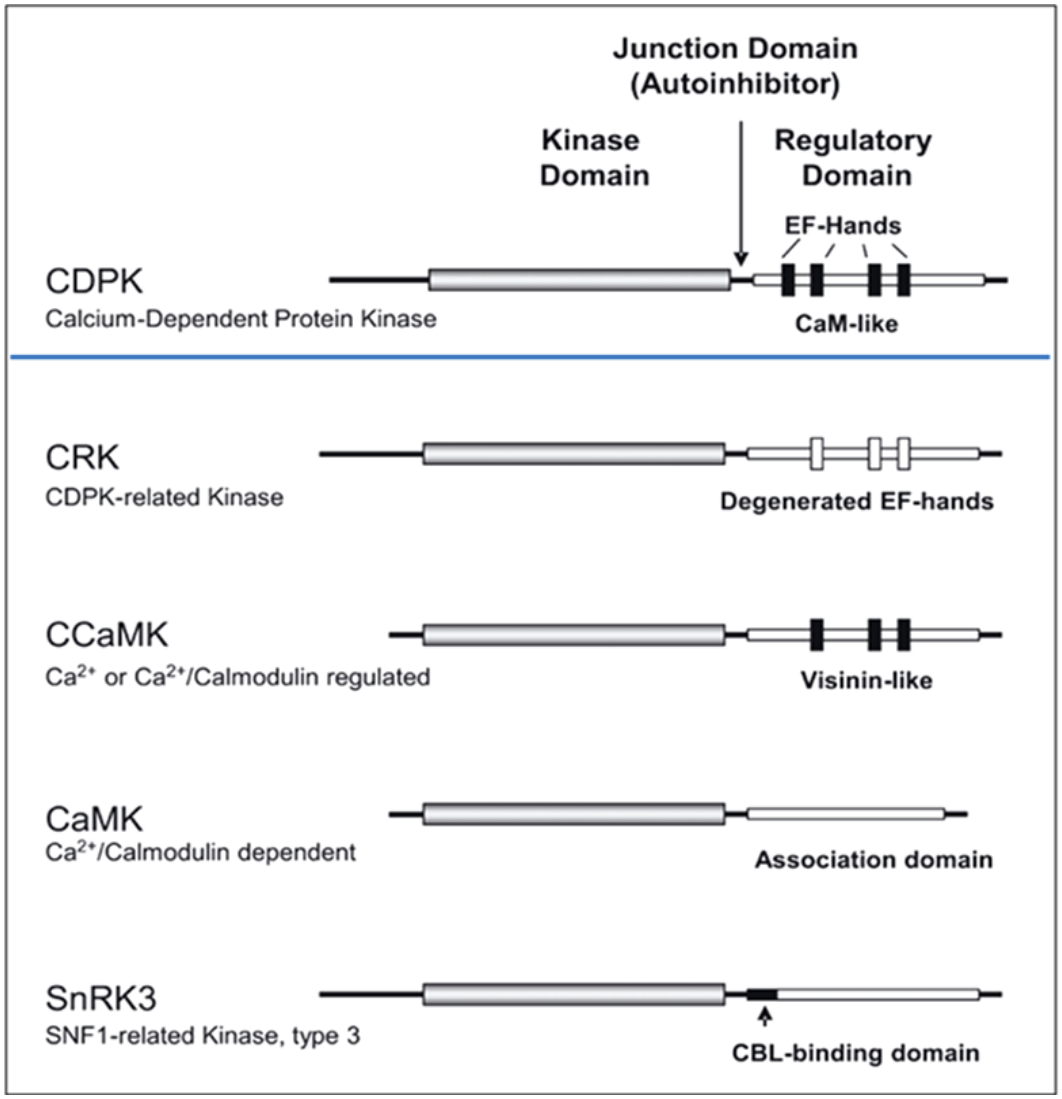

Figure. 1.2. Domain structures of Ca^{2+} regulated protein kinases. The kinases are drawn with the N-terminus on the left with variable length, followed by the kinase domain, autoinhibitory domain and Ca/CaM regulated domains on the far right (modified from Harmon, 2003, Gravit Space Biol Bull 16: 83-90).

modification sites for myristoylation and palmitoylation, which are responsible for their membrane association.

Adjacent to the N-terminal domain is a typical serine-threonine protein kinase domain, which is activated upon Ca^{2+} binding. The kinase domain is a well conserved domain. The Arabidopsis CPKs have overall 44%-95% sequence identities, but with almost 100% identities in the region of the active site (Cheng et al., 2002). This kinase domain is further divided into 12 sub-domains as is typical of eukaryotic Ser/Thr protein kinases. The Lys residue in sub-domain II is the conserved ATP binding site. Mutation of this site in AtCPK1 (K40M) abolishes kinase activity (Sheen, 1996).

An autoinhibitory domain, called the junction domain, lies between the kinase domain and the CaM-like domain. This acts as a switch and regulates the kinase activity of a given CPK. This 31 residue long sequence is rich in basic amino acids, contains a –X-X-S/T phosphorylation site, and acts as a pseudosubstrate (Harmon et al., 1994; Harper et al., 2004). This pesudosubstrate region binds to the active site and keeps the kinase in an inactive form in the absence of sufficient Ca^{2+} concentrations. In presence of Ca^{2+}, the autoinhibitory domain binds to the CaM-like domain and promotes activation of kinase activity. How the autoinhibitory domain regulates activity of CPKs is described below in more detail.

The C-terminal domain of a CPK contains a CaM-like domain (CaMLD) with EF hands for Ca^{2+} binding. Most Arabidopsis CPKs contain four EF hands, but the number can vary between one to four, depending upon the isoform. The EF hands at positions 1 and 2 are closer to the autoinhibitory domain and show a higher degree of conservation

when compared with EF hands at positions 3 and 4. The EF hand at position 4 is the least conserved (Cheng et al., 2002).

Each EF hand comprises two α helices about 10 amino acids each that lie on either side of a loop of 13 amino acid residues. Binding of a single Ca^{2+} to the loop domain leads to a conformational change in the protein (Zhang and Yuan, 1998). Thus, the EF hands are involved in sensing the signal through Ca^{2+} binding and further transduce it through a conformational change which influences the ability of the protein or domain to interact with other proteins and domains (Kawasaki et al., 1998). Following the CaMLD, there is a short amino acid sequence of variable length. However, not much is known about the role of this small variable domain or even whether this should be regarded as a part of the CaMLD.

Sub-cellular Localization of CPKs and Role of Post Translational Lipidation

CPKs have a wide range of sub-cellular distributions. They are found both as soluble forms and as different membrane bound forms at the plasma membrane or on membranes of peroxisomes, ER, or mitochondria (Harper and Harmon, 2005). Collectively, the wide distribution of isoforms allows their access to potentially hundreds of proteins for interactions and/or as potential substrates, while maintaining isoform specific responses.

Most CPKs have myristoylation and palmitoylation sites at the N-terminus (27 out of 34 in Arabidopsis). The Gly residue at the second position has been shown to be a

site for myristoylation in many proteins. In several organisms myristoylated proteins are targeted to membranes, where they can become involved in signaling processes through protein-protein interactions (Towler et al., 1988; Nadolski and Linder, 2007). Myristoylation is an irreversible post translational lipid modification formed by an amide linkage between myristic acid (14 C) and Gly. Several CPKs such as AtCPK2 (Lu and Hrabak, 2002), CpCPK1 (zucchini; Ellard-Ivey et al., 1999), OsCPK2 (rice; Martin and Busconi, 2000), and McCPK1 (ice plant; Chehab et al., 2004) have myristoylation sites required for targeting to the plasma membrane or ER membranes.

Another type of common post translational modification in CPKs is S-acylation or palmitoylation. Palmitoylation involves covalent addition of palmitic acid (16 C) to a cysteine residue by a thioester linkage. In Arabidopsis, the 27 CPKs that have a potential myristoylation site also have one or more palmitoylation sites at positions 3, 4 or 5, except for AtCPK3 (see below; Cheng et al., 2002). Palmitoylated proteins are less strongly anchored to membranes than are myristoylated proteins. Thus, for proper membrane association of palmitoylated proteins, additional prenylation or myristoylation is generally required (Hemsley and Grierson, 2008).

Protein palmitoylation is a reversible process modified by two enzymes, protein acyl transferase (PAT) and acylthioesterase (Nadolski and Linder, 2007). PAT catalyzes the addition of palmitate to the substrate protein, whereas acyl thioesterase cleaves the thioester bond and removes palmitate. Hence, a single protein can go through repeated cycles of palmitoylation and depalmitoylation (Nadolski and Linder, 2007; Hemsley and Grierson, 2008). As a result of this modification cycle, palmitoylated proteins such as

plant GTPases and human Ras proteins are known to cycle intermittently between membranes and soluble micro-domains (Nadolski and Linder, 2007). Several proteins that undergo myristoylation and palmitoylation have been shown to function in stress signaling for pathogen responses, innate immunity, calcium signaling and G protein signaling pathways.

Both myristoylation and palmitoylation are likely important for sub-cellular localization of many CPKs. Dammann et al., 2003) have shown that out of 9 AtCPKs studied, those which have a myristoylation site and at least one palmitoylation site (AtCPK1, AtCPK7, AtCPK8, AtCPK9, AtCPK16, AtCPK21, AtCPK28) are localized to the plasma membrane, whereas AtCPK3 and AtCPK4 lack palmitoylation sites, or both myristoylation and palmitoylation sites, respectively, and occur as soluble proteins with nuclear and cytosolic localization. OsCPK14 also shows a cytosolic localization due to the lack of both myristoylation and palmitoylation sites (Zhang et al., 2005).

Regulation of CPK Activity

Ca^{2+} is the primary regulator of CPK activity. Different Ca signatures activate different CPKs, but the relationship between a specific type of Ca signature and the targeted CPKs is not clear. Activation of a particular CPK is also dependent on the number of EF hands in that isoform. At low concentrations of Ca^{2+}, or in its absence, the autoinhibitory (i.e. junction) domain acts as a competitive inhibitor of the protein kinase domain. The CaM-like domain typically has two lobes which contain two EF hands

each, as shown in (Fig. 1.3). At lower Ca^{2+} concentrations, Ca^{2+} binds to the EF hand at the C-terminal lobe of the CaM-like domain, but does not induce any conformational change in the autoinhibitory domain (Harper et al., 2004). As the Ca^{2+} concentration increases, Ca^{2+} binds to the N-terminal lobe of the CaM-like domain, which then induces a conformation change that removes the autoinhibition. This allows the kinase domain to have catalytic activity and phosphorylate target proteins (Fig. 1.3). NMR studies of AtCPK1 have revealed that Ca^{2+} binding constants of AtCPK1 are 30 nM and 0.6 μM for the C-terminal and N-terminal lobes, respectively (Christodoulou et al., 2004). Similar observations from other plants suggest that as Ca^{2+} concentration spikes, both lobes of the CaM-like domain must be saturated in order to activate CPKs (Hegeman et al., 2006). Variations in both the number of EF hands and the domain amino acid sequences lead to differential affinities for Ca^{2+} binding. Each EF hand is capable of binding one Ca^{2+} and hence, the numbers of EF hands affect the Ca^{2+} binding ability of that protein. The regulation of CPK activity also depends upon the type of substrate available. For example, CPKα from soybean has a K_d of 50 μM for Ca^{2+} binding in the absence of any substrate, but in the presence of substrate the Ca^{2+} binding affinity increases more than 10-fold (Harmon et al., 2000).

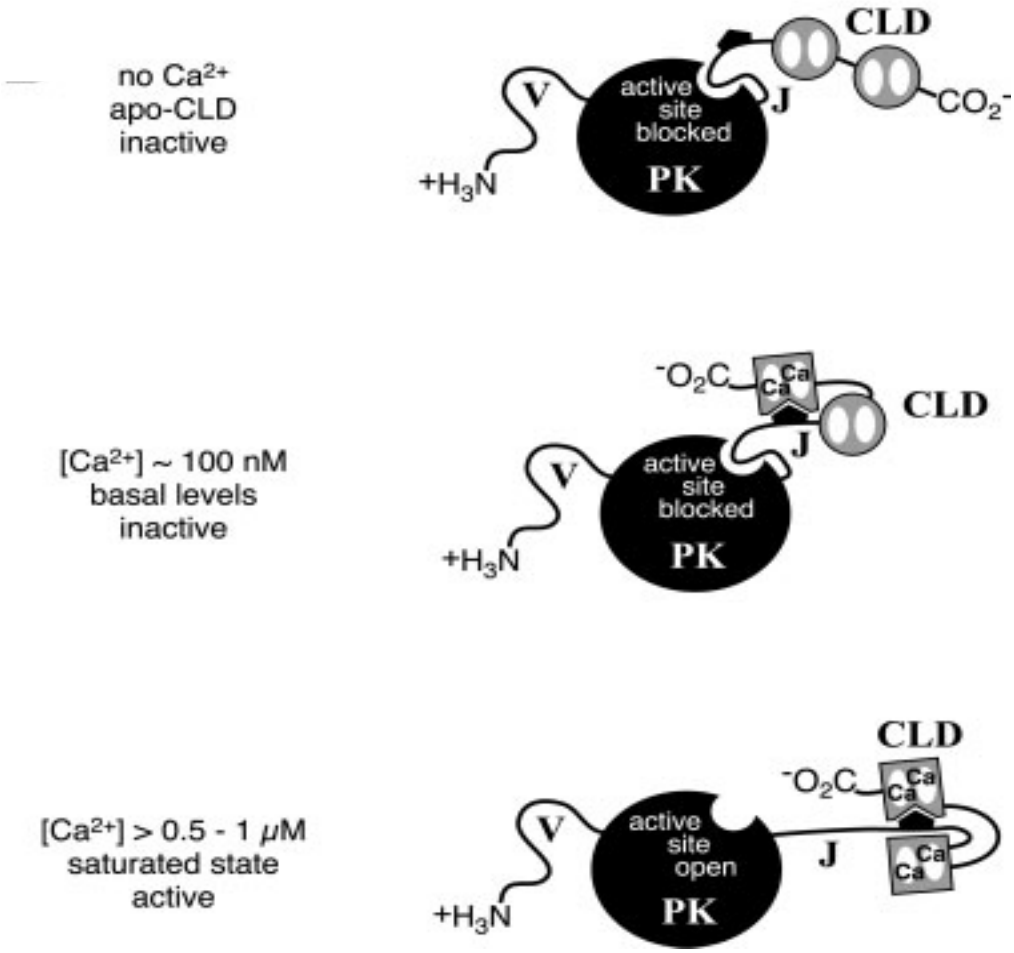

Figure 1.3. Regulation of CPK activity. V indicates the N-terminal variable domain. PK is the protein kinase domain. J is the junction or autoinhibitory domain. CLD is the CaM-like domain, with white ovals representing each EF hand (From Hegeman et al., 2006, Proteomics 6: 3649-3664)

In addition to Ca regulation, phosphorylation and dephosphorylation can also regulate the activity of CPKs. Autophosphorylation refers to the phosphorylation of possible Ser/Thr residues at potential regulatory sites by the active protein kinase domain. So far it has not been confirmed conclusively that autophosphorylation alone is sufficient for CPK activation. In some CPKs autophosphorylation is required for substrate phosphorylation whereas in CCaMKs autophosphorylation increases the affinity towards CaM binding (Harper et al., 2004). *In vitro* phosphorylation does activate a ground nut (*Arachis hypogea*) CPK (Shah and Singh, 1995). Only four regulatory phosphorylation sites have been identified that are specific to serine/threonine residues in studies involving tomato, tobacco and ice plant CPKs (Rutschmann et al., 2002; Glinski et al., 2003; Witte et al., 2004). Arabidopsis and Plasmodium CPKs have potentially revealed 31 autophosphorylation sites, of which more than half are located in the NTV domain (Hegeman et al., 2006). A possible physiological role of these autophosphorylation sites is still ambiguous. For one, there are limited available knockout phenotypes of CPKs in the superfamily which might be examined by complementation studies. However, dephosphorylation also has an important role in regulating activity of CPKs. Though most CPKs are activated by phosphorylation, one wing bean CPK is inactivated by autophosphorylation and then activated by a phosophoserine phosphatase (Ganguly and Singh, 1999).

The biochemical characterization NtCPK2 from *Nicotiana tobacum* shows that in a transient expression assay it is activated in response to fungal elicitors and osmotic stress (Romeis et al., 2001). This activation was attributed to phosphorylation of

NtCPK2, as seen by a mobility shift assay and by increased enzyme activity (Romis et al., 2000). The activation response to fungal elicitors is even more profound as compared to osmotic stress, suggesting there occurs variable regulation of CPK activity in a stimulus-dependent manner. Biochemical evidence has shown that certain phospholipids can enhance Ca^{2+} dependent phosphorylation of CPKs by several-fold from plant species such as oat (*Avena sativa*, Schaller et al., 1992), Arabidopsis (AtCPK1, Harper et al., 1993; Binder et al., 1994), carrot (*Daucus carota*, Farmer and Choi, 1999), and maize (ZmCPK11, Szczegielnial et al., 2005). However, there occurs specificity between CPKs and a particular type of phospholipid molecule. For example, Szczegielnial et al. (2005) reported that maize CPK (ZmCPK11) shows phosphoregulation in response to treatment with phosphatidic acid, phosphatidylserine, and phosphatidylinositol, but not by phosphatidylcholine or l-α-lysophosphatidylcholine. Even though ZmCPK11 can be activated by phospholipids, whether phospholipid regulation of any ZmCPKs might have physiological significance needs to be further clarified.

CPK Substrates and their Specificity

Though CPKs occur as multigene families in a given plant and are thought to possess functional redundancies, nonetheless they are generally multifunctional kinases involved in a variety of cellular and developmental processes. Using synthetic peptides and two spinach CPKs (SoCPK I and SoCPK II), two different target phosphorylation motifs have been identified (Cheng et al., 2002, and references there in). The maximum

phosphorylation was achieved by the motif $_{-6}$- φ $_{-5}$-X$_{-4}$-Basic$_{-3}$-X$_{-2}$-X$_{-1}$-S-X$_{+1}$-X$_{+2}$-X φ $_{+4}$-Basic$_{+5}$ which is similar to a site recognized by cauliflower and maize CPKs and also is similar to mammalian protein kinase C (φ is a hydrophobic residue, S is Ser for phosphorylation, and X is any residue; Cheng et al., 2002). The other site phosphorylated by spinach SoCPK I and SoCPK II is Basic$_{-9}$-Basic$_{-8}$-X$_{-7}$- Basic$_{-6}$-φ$_5$-X$_{-4}$-X$_{-3}$-X$_{-2}$-X$_{-1}$-S-X$_{-1}$-Basic$_{-2}$ (Huang et al., 2001). Since this second motif cannot be phosphorylated by SnRK1, it is thought to be highly specific to CPKs (Huang and Huber, 2001). This motif has been useful to identify other CPK substrates such as spinach plasma membrane aquaporin PM28A, ACC synthase, RNA polymerase β chain, a protein kinase, a splicing factor, and two disease resistance gene homologues (Cheng et al., 2002).

Apart from the above examples, many CPK substrates have been identified through *in vivo* phosphorylation studies. Some examples of these substrates are phenylalanine ammonia lyase, 14-3-3 proteins, actin depolymerizing factor, a 26S proteosome regulatory factor, and ion and water transport proteins such as a plasma membrane proton pump, an ER associated Ca^{2+} pump, and a guard cell potassium channel (see review by Harper and Harmon, 2005). *In vitro* studies using yeast two hybrid screens have also indentified CPK substrates. Zhu et al. (2007) have shown that both AtCPK4 and AtCPK11 phosphorylate two ABA responsive transcription factors, ABF1 and ABF4.

Functional specificities of CPKs can be achieved through different mechanisms. One such mechanism involves their different sub-cellular localization. First, CPKs are

present at diverse locations such as the plasma membrane, ER, nucleus, peroxisomes, and cytosol. This allows them to collectively interact with a vast array of other proteins involved in cellular signaling and can lead to extensive crosstalk. For example, both AtCPK4 and AtCPK11 are localized to the cytoplasm and nucleus (Dammann et al., 2003; Milla et al., 2006). This dual localization allows these CPKs to produce early, as well as delayed responses to ABA (Zhu et al., 2002). In guard cells, cytosolic CPK4 and CPK11 might more easily mediate a rapid response such as stomatal closing by sensing Ca^{2+} and phosphorylating downstream messengers already in place, whereas nuclear CPK4 and CPK11 can phosphorylate regulators of gene expression such as the transcription factors ABF1and ABF4 (Zhu et al., 2006). A second mechanism for achieving functional specificity can involve diversification of the phosphorylation motifs. Apart from the two classic motifs mentioned above, four other motifs have been identified. This indicates that other unique substrates might be phosphorylated by CPKs in varying ways (Harper and Harmon, 2005).

Biological Functions of CPKs

Although it has been more than 25 years since the identification of CPKs, a full understanding of the function of individual CPK in a given species remains an elusive goal. Biochemical characterizations using *in vivo* phosphorylation assay have identified several protein substrates which indicate that CPKs are involved in diverse signaling pathways that affect many aspects of plant growth. One of the obstacles in characterizing

individual CPK function is the apparent functional redundancy which occurs among isoforms of the CPK gene family. Reverse genetics is one approach in which mutants and overexpression lines for specific isoforms can be used to characterize single or multiple CPKs.

Growth and development

Ca^{2+} affects numerous aspects of growth and development. Some CPKs have been shown to affect specific organ growth and development, and also overall plant growth. Sandalwood CPK shows differential activity in different developmental stages of embryogenesis (Anil and Rao, 2000; Anil et al., 2003). The lack of a functional rice CPK (SPK), which is exclusively expressed in endosperm of immature rice seeds, leads to watery seeds with low levels of starch. SPK has an apparent role in endosperm development (Asano et al., 2002). Other rice CPKs such as OsCPK2 and OsCPK11 are also associated with seed development as their expression is associated with specific developmental stages of seed formation (Frattini et al., 1999). One CPK located on the Y chromosome of liverwort, a primitive plant, is constitutively expressed in the male sex organ, inferring that it has role in reproduction (Nishiyama et al., 1999).

Pollen tube growth is a specialized case of organ development. A maize CPK is expressed in late stages of pollen development, perhaps having a role in pollen germination and/or pollen tube growth (Estruch et al., 1994). Increased CPK activity in the pollen tube of *Agapanthus umbellatus* results in reorientation of the pollen tube

(Moutinho et al., 1998). CPKs are also involved in the self incompatibility response. A CPK from *Nicotiana alata* phosphorylates an RNase in the style, suggesting it might have a role in regulation of self incompatibility by blocking enzyme activation and thus allowing tube growth (Kunz et al., 1996). Yoon et al. (2006) have identified two petunia CPKs that are involved in pollen tube growth (PiCPK2) and the regulation of polarity. A recent study by Myers et al. (2009) has shown that two Arabidopsis CPKs, AtCPK17 and AtCPK34, have roles in controlling pollen transmission and pollen tube growth. The single knockout mutants lack a detectable phenotype, but the homozygous double knockout mutant plants are sterile which suggests there is functional redundancy between these two CPKs. Both CPKs are also involved in tropism of pollen tube growth as the tubes fail to locate and fertilize the ovules in the double mutant.

CPKs are involved in different signaling pathways in plants which affect developmental processes such as organ growth, flowering and even nodulation through selective activity of certain CPKs. In potato, StCDPK1 and StCDPK3 are differentially and sequentially expressed in early stages of tuber formation (Raices et al., 2001 and 2003). StCDPK3 is only expressed early in elongating stolons whereas StCDPK1 is expressed later in the swollen tips of stolons that differentiate into tubers. In Arabidopsis, AtCPK1 promotes plant growth as inferred by an overexpression phenotype (Yu et al., 2007). In grape berry, CPK1 is primarily expressed in the fleshy portions of the berry and later in the seeds (Yu et al., 2006). In tobacco, NtCPK4 is expressed spatiotemporally in vegetative and reproductive tissues during their growth and development (Zhang et al., 2005). However, NtCPK1 acts as a repressor of shoot growth

in a gibberellic acid dependent manner (Ishida et al., 2008). MtCPK1 from *Medicago truncatula* modulates root hair growth by affecting the expression of many genes that are involved in cell wall synthesis and cell expansion (Ivashuta et al., 2005). A cucumber CPK is known to have a complex role in adventitious root formation, mediating crosstalk with auxin, nitric oxide and Ca^{2+} (Lanteri et al., 2006). MtCPK3 also affects legume nodulation and nitrogen fixation, being one factor controlling nodule number in *Medicago* (Gargantini et al., 2006). In fact, two nodule specific proteins, nodulin 26 (Weaver and Robers, 1992) and nodulin100 (Zhang and Chollet, 1997) are phosphorylated by CPKs

Guard cell and stomatal movements

Stomata are small pores on the epidermal surface of plants that are bordered by a pair of modified epidermal cells called guard cells. The guard cells control the shape and size of the stomatal pore to allow differential uptake of CO_2 or release of water. They function as a gateway for the proficient uptake of CO_2 and exchange of other gases and water vapor through the stomatal pore. Stomatal pores close in response to dehydration, water stress, and action of a plant stress hormone abscisic acid (ABA). ABA triggers closing of stomatal pores by inducing increased cytosolic Ca^{2+} concentration in guard cells (McAinsh, 1997; Hamilton et al., 2001). ABA also induces the Ca^{2+} influx which is associated with stomatal opening. To maintain balance between Ca^{2+} and K^{+} ion uptake

during stomatal movements, anions such as Cl^- or malate are exported into vacuoles (Fan et al., 2004).

CPKs have complex regulatory roles in stomatal behavior. Pei et al. (1996) have shown that AtCPK1 (earlier called AK1) significantly activates chloride channels to induce chloride and malate uptake in *Vicia faba* guard cells. Also, in Arabidopsis plants that overexpress AtCPK1, ABA-induced stomatal closing was impaired, leading to less water loss during dehydration (Yu et al., 2007). Together these observations indicate that AtCPK1 regulates ion uptake in guard cells and therefore stomatal movements associated with osmosis. In an opposite regulatory fashion, the overexpression of AtCPK4 and AtCPK11 made Arabidopsis plants hypersensitive to ABA-induced stomatal closing and inhibited stomatal opening (Zhu et al., 2007). Their respective mutants were insensitive to ABA.

Additional regulation of guard cell function occurs also through AtCPK3 and AtCPK6. These both regulate the activities of ABA and Ca^{2+} activated anion channels in guard cells. Mutants of AtCPK3 and AtCPK6 showed disruption of ABA-induced activation of plasma membrane Ca^{2+} permeable channels in guard cells (Mori et al., 2006). These mutants were also insensitive to ABA and Ca^{2+}-induced stomatal closing. Thus, these results provide direct evidence for the function of CPKs in stomatal movements through ABA and Ca^{2+}. Microarray analysis of Arabidopsis guard cells has also shown that other CPKs apart from those mentioned above (CPK11, CPK7 and CPK 13) are also expressed in guard cells and likely have regulatory functions (Leonhardt et al., 2004).

Carbon and nitrogen metabolism

Different experiments have shown that several enzymes associated with carbon and nitrogen metabolism are substrates of particular CPKs. Sucrose synthase (SuSy) and sucrose phosphate synthase (SPS) are key enzymes in carbon metabolism, whereas nitrate reductase (NR) is important in nitrogen metabolism. CPK has been shown to phosphorylate an N-terminal Ser residue of SuSy from many plant species. This phosphorylation site might be CPK specific since mammalian protein kinase A is unable to phosphorylate this Ser residue (Huber et al., 1996; Zhang et al., 1999; Loog et al., 2000).

CPKs from spinach (SoCPK I and SoCPK III but not SoCPKII) can differentially phosphorylate Ser-158 of SoSPS *in vitro,* thus inactivating the enzyme as is thought to occur in the dark. This inactivation depends upon the presence of a Pro residue at the P-4 position (McMichael et al 1995a; 1995b). However, lack of this Pro-4 leads to phosphorylation of SPS, while in Arabidopsis, presence of the Pro avoids phosphorylation and thus inactivation of spinach SPS (Huang and Huber, 2001). Such diverse effects likely reflect different regulatory adjustments that plants use to modulate SoSPS activity.

Spinach SoCPKI and SoCPKII also both phosphorylate and inactivate NR in the dark (Douglas et al., 1998; Bachmann et al., 1996). While these CPKs do modulate activities of SuSY, SPS, and NR by direct phosphorylation, *in vivo* experimental

evidence is needed to understand the physiological significance of these processes among different species.

Hormone signaling

Variations in cytosolic Ca^{2+} concentration lead to perturbations in many metabolic and signaling pathways, which in turn affect hormone signaling pathways. Additionally, plant hormones like auxin, cytokinins, ABA, gibberellins, jasmonic acid, and brassinosteroids are known to influence expression of several CPKs. CPKs can be involved in hormone signaling either by phosphorylating multiple substrates in a particular pathway or by regulating expression of genes that are key candidates of a hormone pathway. The hormones may also regulate expression of CPKs that can act as early response genes activating several downstream components.

Abo-el Saad and Wu (1995) first showed that CPKs are activated by hormone signaling, as much as 10-fold in gibberellin treated rice seeds. Later, Yang et al. (2003) also showed that the transcript level of rice OsCPK13 was increased by treatment with gibberellin. In leaves of chickpea, both transcript and protein activity of CaCPK2are also induced by treatment with gibberellin (Syam Prakash and Jayabaskaran, 2006). Regulation of CPK transcript levels by gibberellin was also shown in tobacco, in which NtCPK4 is induced within 10 minutes following gibberellin treatment, and is restored to basal levels after two hours (Zhang et al., 2005). In aleurone layers of barley seeds,

HvCPK1 is involved in gibberellin-dependent vacuolation and vacuolar acidification through post transcriptional regulation (McCubbin, 2004).

Cytokinins also can alter transcript abundance of several CPKs. In cucumber, CsCPK1 and CsCPK2 both were induced by treatment with a synthetic cytokinin, benzyladinine (BA), resulting in increased kinase activity (Syam Prakash and Jayabaskaran, 2006). Similar results were reported for CsCPK3, which is regulated by BA in an organ specific manner. Expression of CsCPK3 in cotyledons is upregulated after treatment with BA, but in roots its expression is down-regulated, while in hypocotyls it remains unchanged (Ullanat and Jayabaskaran, 2002). A nodulation specific CPK from *Medicago truncatula*, MtCPK3, also shows increased expression in response to cytokinin treatment (Gargantini et al., 2006). MtCPK3 is involved in cortical cell division during formation of nodule primordial.

Other studies have shown additional hormone specific regulation of CPK expression. Induction of VrCPK1 from cuttings of mung bean (*Vigna radiata*, Botella et al., 1996) and alfalfa (*Medicago sativa*) callus suspension cultures was specific to treatment with auxin (Davletova et al., 2001). Brassinosteroids induce rice CPK (Yang and Komatsu, 2001), while jasmonic acid affects expression of potato StCPK2 (Ulloa et al., 2002). Grape berry ACPK1 is induced by ABA, but auxin, cytokinin, gibberellin, and brassinolide do not produce a similar inductive effect (Yu et al., 2006).

Along with target specificity of CPKs by individual hormones, there also occur extensive crosstalk between one or more hormone signaling pathways and their influence on CPK expression. For example, NtCPK1 from tobacco is transcriptionally upregulated

by gibberellin, ABA, as well as cytokinins (Yoon et al., 1999). Rice OsCPK13 is phosphorylated in a gibberellin dependent manner and is upregulated in response to gibberellin, but is down regulated by ABA and brassinolide (Abbasi et al., 2004). Apart from these examples, the expression of many CPKs from different plant species is regulated by ABA either as an ABA specific response or in accordance with other abiotic and biotic stresses.

ABIOTIC AND BIOTIC STRESS SIGNALING

Overview of ABA in Stress Signaling

The phytohormone ABA regulates several developmental processes in plants which include seed maturation and dormancy, seed germination, seedling growth, flowering, and stomatal movements. Apart from these developmental processes, ABA also regulates responses and adaptation to the changing environment such as drought, salt stress, osmotic stress, heat, wounding, and pathogenesis. ABA was first discovered in the 1960s from young cotton bolls and from sycamore leaves. It was given the names dormin or abscissin due to its occurrence at abscission areas. Understanding the role of ABA in seed development, synthesis of storage proteins and lipids, achieving desiccation tolerance, establishing seed dormancy, inhibiting embryonic germination, and breaking dormancy during germination have been very important in controlling pre-harvest germination in many crop species as it affects quality and yield in grain crops. Moreover,

ABA signaling through stimulus specific biosynthesis and regulation of gene expression, offers resistance to plants during many adverse environmental conditions such as drought, cold, high salinity as well as pathogenesis. Thus, ABA is considered to be a stress hormone involved in abiotic and biotic stress signaling in plants. Acquired desiccation tolerance was important to the evolution of land plants which relied on ABA dependent processes (Rensing et al., 2008).

ABA is produced from a 5 carbon precursor, isopentenyl pyrophosphate (IPP). In chloroplasts, IPP is used to produce β-carotene, which undergoes hydroxylation to produce zeaxanthin. Repeated isomerization steps then lead to formation of 9-cis-violaxanthin/neoxanthin, which is finally converted into xanthoxin through deoxygenase activity of the NCED gene (VP14 in maize and NOT in tomato). The deoxygenase activity is thought to be a rate limiting in ABA production. Further xanthoxin is converted to ABA in the cytoplasm through abscisic aldehyde or abscisic alcohol intermediates (Wasilewska et al., 2008; and references therein). Once synthesized, the inactive form of ABA is transported to various plant organs as glucose ester conjugates to be stored in vacuoles and in the apoplastic space until activation. For example, ABA-esters from vacuoles and the apoplastic space is transported to the ER and converted into the active form in response to dehydration (Lee et al., 2006). Water stress also leads to a four-fold increased conversion rate of active ABA conjugates in Arabidopsis leaves (Wasilewska et al., 2008).

Under normal conditions, ABA is produced in small quantities at the apical meristem, in veins, hydathodes of cotyledons, in guard cells, root columella cells, and in

the quiescent center of the root. In response to water stress treatment of roots, ABA production is enhanced in leaves and in the vasculature. It remains unclear whether ABA is normally synthesized in leaves and is then transported to roots and other organs, or if it is synthesized in roots and then transported to aerial organs (Wasilewska et al., 2008). The response to stress begins with sensing of a stress signal and amplification of the stress signal through second messengers, followed by subsequent changes in physiological and metabolic processes such as phosphorylation and dephosphorylation of target proteins, or activation of gene expression of stress responsive genes, which then further affect metabolic processes. ABA signaling involves a large array of genes including many transcription factors and response regulators that bind to the ABA response elements.

Perception of stress, or stress causing agents, is the first step in stress signaling. Apart from binding a ligand to a specific receptor, other elements that can lead to recognition of a stimulus are changes in turgor, membrane strain, changes in the shape of cells, and molecular crowding (Verslues and Zhu, 2005). For several decades, the efforts to identify receptors for ABA have met with limited success. In the past few years, several putative ABA receptors have been reported. First, the RNA binding protein flowering time control protein A (FCA) was reported to be a possible receptor by Razem et al. (2006). A second possible ABA receptor belonging to the Mg-chelatase family, GUN5, was identified through high affinity ABA binding (Shen et al., 2006). As a third possibility, G protein-coupled receptor 2 (GCR2) was reported to be a membrane localized receptor for ABA perception (Liu et al., 2007). However, the lack of *gun5* and

gcr2 alleles with strong ABA insensitivity makes it difficult to fully appreciate GUN5 and GCR2 as ABA receptors. Apart from these, ABI4 was also considered as a potential candidate for the ABA receptor (Koussevitzky et al., 2007). Recently two independent studies have reported alternative ABA receptors from Arabidopsis. Ma et al. (2009) have identified the regulatory component of ABA receptor (RCAR) as a potential ABA receptor through interactions with two type 2C protein phosphatases (PP2C) ABI1 and ABI2. RCAR1 binds to ABA with high affinity and leads to ABA dependent inactivation of ABI1 and ABI2, two negative regulators of ABA signaling. Another study involving chemical genetics by Park et al. (2009) has reported that pyrabctin resistant 1 (PYR1) from Arabidopsis binds to ABA and is able to inhibit PP2C activity *in vivo*. PYR1 and the PYR-like proteins (PYLs) are the ABI interacting proteins called RCARs (Ma et al., 2009). The PYR/PYL complex regulates the ligand specificity for PP2C interactions and inhibition (Park et al., 2009).

Further transduction of ABA signaling largely depends upon activities of many transcription factors and response genes. Many ABA signaling components have been identified from mutants that are either hypersensitive or insensitive to ABA, or that are deficient in ABA production. Early studies by Koornneef et al. (1989) identified ABI1 and ABI2 mutants as having a partial ABA insensitive phenotype. Later, Sheen (1996) showed that both ABI1 and ABI2 act as negative regulators of ABA signaling. Three other ABA insensitive genes, ABI3, ABI4, and ABI5 were later identified as transcription factors for seed specific and ABA inducible genes (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000).

Among these, ABI3 is an ortholog of VIVIPAROUS 1 (VP1) in maize, while ABI4 is related to the AP2 type transcription factors which are closely related to drought response element (DRE) binding factor (DREB). Two other transcription factors that are similar to ABI5, AREB1 (ABF2) and AREB2 (ABF4) bind to ABA response elements (ABRE) in the promoters of ABA response genes and activate their expression (Choi et al., 2000).

There are four *cis* acting elements that are found in almost all ABA response genes as binding sites for transcription factors. These are G-box elements known as ABRE, the functional equivalent of ABRE called ABRE coupling element ABRE-CE (similar to DRE), RY/Sph elements, and the recognition sequences of MYB and MYC type of transcription factors (Busk and Pages, 1998; Rock et al., 2000). ABREs are bound by bZIP type transcription factors, whereas RY/Sph elements are bound by B3 type proteins (Finkelstein et al., 2002). One transcription factor can be activated by more than one type of stress. For example, MYB family members are activated both by salinity and drought through ABA intermediates (Nakashima and Yamaguchi-Shinozaki, 2006).

Other *cis* acting elements involved in both ABA-dependent and ABA-independent stress signaling are the DRE elements or C-repeats elements (CRT). The DREB type of transcription factors are involved in cold stress through DREB1/CBF and in drought stress through DREB2, by affecting expression of genes that have DRE/CRT elements in their promoters (Knight and Knight, 2001). Thus, the use of different types of transcription factors belonging to multigene families allows both specificity as well as considerable flexibility by ABA/stress signaling pathways. The complexity of the

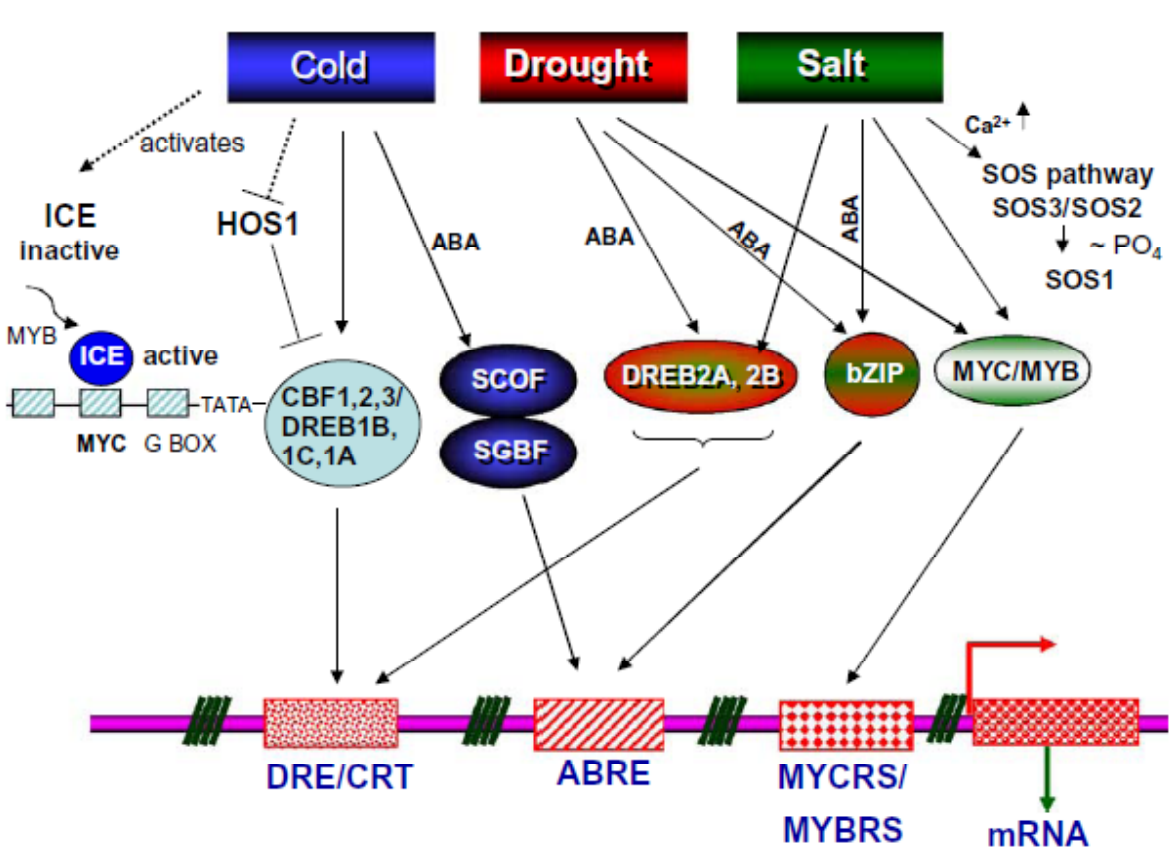

Figure 1.4. Schematic representation of the regulatory network of gene expression in response to cold, salinity, and drought. Ovals represent the transcription factors whereas squares represent the response genes. Figure borrowed from Mahajan and Tuteja, 2005 Arch Biochem Biophys 444:139-58. HOS1, high expression of osmotically responsive 1, ICE1, inducer of CBF expression 1; DREB, DRE binding factor; CBF, C-repeat/DRE binding factor; CRT- C-repeats element; SCOF, Soybean cold inducible factor; SGBF, Soybean G-box binding factor; DREB, drehydration response element binding factor; DRE, dehydration responsive element; ABRE, ABA-responsive element; bZIP, basic leucine zipper.

regulatory network in response to osmotic stress caused by salinity, drought, and cold stress is shown as currently understood in Fig. 1.4. As shown in Fig. 1.4, the plants have several different mechanisms to respond to salt stress; throught SOS signaling pathways by involving CBLs, ABA independent mechanism involving DREB and MYC/MYB type of trsnascription factors or in an ABA dependent way using bZIP type of transcription factors. These transcription factors then affect the genes that have the binding sites for the specific transcription factors leading to the specificity of the response.

The ABA response genes are divided into two groups, the early response genes and the late response genes. The early response genes belong to GTP binding proteins, phospholipases such as PLC and PLD, protein kinases, and protein phosphatases. The late response genes involve transcriptional regulation through interaction with various transcription factors and mostly lead to adaptive responses associated with the given stress. One of the most studied genes involved in ABA as well as dehydration belongs to the family of response to desiccation (RD). The promoter sequence of RD29A has both the ABRE and the DRE elements and its expression is enhanced in response to ABA or drought, whereas RD29B only has ABRE elements and is specific to stress signaling through ABA (Yamaguchi-Shinozaki and Shinozaki, 1993). Recent advances in transcript profiling have identified a number of genes that are involved in stress signaling. Some of the stress regulated genes include responsive to ABA (RABs), enhanced responses to ABA (ERA), both ABA-dependent and independent cold response genes (COR), cold inducible (KIN) genes, and early response to dehydration (ERD) genes (Ishitani et al., 1997; Yamaguchi-Shinozaki, 2000).

ABA-independent salt stress signaling involves the CBL type of Ca^{2+} sensors. In response to salt stress, the Ca signatures produced in the cytosol activate the CBL type of Ca sensors through the SOS pathway. In response to this salt specific Ca signature, SOS3/CBL4 interacts either with SOS2/CIPK24, or with CBL10 (Kim et al., 2007; Quan et al., 2007). The SOS3/CBL4-SOS2/CIPK24 complex then regulates the activity of SOS1, a Na^+/H^+ antiporter associated with the plasma membrane. The specific function of SOS2/CIPK24-CBL10 is not clear yet, though it is thought to have a role in maintaining ion homeostasis by interacting with proteins on the vacuolar membrane (Luan, 2009). Since Ca^{2+} acts as a second messenger in response to several abiotic and biotic stresses, many CPKs could be involved in stress signaling pathways. In general, the evidence for their involvement comes mostly from changes in their transcript levels, from changes in their protein kinase activities, and/or from altered expression of genes in stress signaling pathways.

Early studies using Arabidopsis plants show that AtCPK1 and AtCPK2 are induced by drought and high salinity, but not in response to cold or heat stress (Urao et al., 1994). These responses show specificity of these CPKs towards osmotic and salt stress. Sheen (1996) demonstrated that AtCPK10 and AtCPK30, but not AtCPK1 and AtCPK11, activate a stress and ABA responsive promoter in a protoplast transient assay. She proposed that CPKs have specificity towards a given stress signaling pathway. Yoon et al. (1999) have shown that NtCPK1 transcript is induced by salt treatment and that the degree of induction depends upon the treatment time. In rice, cold and salt treatments lead to enhanced transcript abundance for OsCPK7 (Saijo et al., 2000). Further,

transgenic rice plants overexpressing OsCPK7 have increased resistance to cold, drought, and salt stress which corresponds to increased OsCPK7 mRNA expression levels. These overexpression transgenics showed enhanced expression of salt and drought inducible rice genes such as Rab 16A, OsLEA 3, and Sal T, but did not show alterations in several cold inducible genes. Salt/drought stress and cold stress might have independent downstream targets (Saijo et al., 2000). The mung bean VrCPK1 is upregulated by mechanical strain treatment (downward bending) within 30 min of stress treatment and remains upregulated for three hrs (Botella et al., 1996).

Comprehensive studies involving rice CPKs have shown that several rice CPKs have multiple stress response cis elements in their promoters, which are involved in different stress responses. OsCPK6, OsCPK13, and OsCPK25 are upregulated by dehydration, cold, and heat shock, respectively, whereas OsCPK17 is down-regulated by salt stress, cold and dehydration (Wan et al., 2007). In Arabidopsis both AtCPK4 and AtCPK11 are involved in ABA dependent abiotic stress signaling. AtCPK4 and AtCPK11 mutants are insensitive to ABA during seed germination, seedling growth, and stomatal movements. However, the mutants also show increased sensitivity to salt and drought stress. The *cpk4/cpk11* double mutant shows increased sensitivity to ABA and salt in seed germination assay when compared with single mutants, this indicates possible functional redundancy of these two CPKs (Zhu et al., 2007).

Several CPKs from different species are also involved in pathogenesis and defense related responses. In tobacco, CPK activity was induced by 10 to 200-fold in response to pathogen elicitors (Romeis et al., 2000; Romeis et al., 2001). Further,

Romeis et al. (2001) have shown that NtCPK2 and NtCPK3 are crucial in mediating the hypersensitive response to the fungal elicitor. Both NtCPK2 and NtCPK3 are up-regulated in response to fungal elicitation and silencing of these CPKs leads to a reduced hypersensitive response (HR) such as necrosis. In tobacco leaves, NtCPK1 mRNA levels are stimulated both by abiotic and biotic stresses including fungal elicitors, such as chitosan, a fungal cell wall component (Yoon et al., 1999). Further investigation of NtCPK1 expression has shown that NtCPK1 transcript is also upregulated by wounding and salt within two hrs (Yoon et al., 1999). Knockdown mutants of NtCPK1 in *N. benthamiana* show spontaneous necrotic lesions and enhanced levels of defense related marker genes, implicating its involvement in plant defense (Lee et al., 2003). A similar response has been observed in maize and tomato, in which ZmCPK11 and LeCPK1 both are upregulated in response to wounding. But in tomato, only the wounded leaves show up-regulation of LeCPK1 (Chico et al., 2002), while in maize, both the wounded and neighboring leaves show an induction response suggesting its involvement in systemic acquired resistant (Szczegielnial et al., 2005).

Reactive oxygen species (ROS) are generated at the plasma membrane in response to pathogen attack and act to limit further damage and spread of pathogens. In tobacco, plants expressing a constitutively active form of NtCPK2 also respond to mild abiotic stress stimuli with HR-like symptoms and also show increased production of ROS and induction of defense related genes. On the other hand, ectopic expression of NtCPK3 does not lead to induction of defense response genes and an HR-like response (Ludwig et al., 2005), suggesting there is specificity for each CPK. Injection of *Agrobacterium* in a

transient assay using leaves shows that NtCPK2 is involved in producing a strong biotic stress response through induction of ROS and pathogen response genes leading to cell death (Romeis et al., 2001). Also, the transient expression of NtCPK2 leads to accumulation of stress hormones such as jasmonic acid, ethylene, and 12-oxo-phytodienoic acid that are produced in response to strong biotic stresses (Ludwing et al., 2005). AtCPK1 from Arabidopsis is closely related to NtCPK2 and is also associated with pathogen defense. In a protoplast transient assay, overexpression of AtCPK1 leads to increased NADPH activity and ROS production (Xing et al., 2001). In potato, StCPK4 and StCPK5 are also involved in regulating pathogen response through production of ROS. These CPKs regulate an oxidative burst through phosphorylation of two potato NADPH oxidases in response to pathogen attack (Kobayashi et al., 2007). AtCPK3 and AtCPK6 are involved in ABA induced stomatal closing and are indirectly involved in guard cell ROS signaling by affecting activities of Ca^{2+} channels and an NADPH oxidase (Mori et al., 2006).

Recently several monocot species have shown that CPKs have a role in pathogenesis. In wheat, twelve out of twenty CPKs show a transciptional response to powdery mildew treatment (Li et al., 2008). In barley (*Hordeum vulgare*), two CPKs were also shown to have antagonistic effects that are pathogen specific. In a transient expression assay, constitutively active HvCPK3 promotes fungal entry in leaf mesophyll cells, in contrast to HvCPK4. HvCPK4, but not HvCPK3, induces cell death in infected areas in a kinase-dependent manner (Freymark et al., 2007). In orchid, *Phalaenopsis*

amabilis PaCPK1 was transcriptionally upregulated in response to pathogenesis, wounding, and low temperatures (Tsai et al., 2007).

Several observations indicate that many CPKs are involved in more than one type of stress signaling and thus have distinct, as well as overlapping functions, in abiotic and biotic stress signaling. A classic example for involvement of CPKs in multiple stress signaling is seen in wheat. Li et al. (2008) have shown that seven of the twenty wheat CPKs were regulated by ABA, five by salt, eight by H_2O_2, seven by cold, and fourteen were regulated at least by one or more stresses suggesting there is extensive crosstalk between wheat CPKs and various stress signaling pathways. Also, expression of TaCPK4 is regulated by salt, cold, ABA, gibberellin, fungal pathogen, and powdery mildew treatments. Both chickpea CaCPK1 and CaCPK2 are induced by salt, but CaCPK1 and not CaCPK2 is induced in response to fungal infections, while CaCPK2 and not CaCPK1 is induced by dehydration (Syam Prakash and Jayabaskaran, 2006). These results show that both CPK1 and CPK2 from chickpea might have redundant function in salt stress signaling, but maintain specificities towards dehydration stress and pathogenesis. Also, five rice CPKs (OsCPK10, 12, 13, 15 and 21) are upregulated in response to desiccation, but their expression remains largely unaffected by treatment with salt (Ray et al., 2007). In tobacco, expression of constitutively active NtCPK2 triggers biotic and abiotic stress response.

As our understanding of the functions of CPKs improves, the role of CPKs in abiotic and biotic stress signaling will become clearer. Many experiments mentioned above show an indirect involvement of CPKs in stress signaling, the majority of which

are based on transcriptional regulation of CPKs in response to a given stress. Due to possible redundancy among several isoforms as well as complexity in their specificity, it has been a challenging task to understand the function of CPKs even in model species such as Arabidopsis and rice. Single and multiple loss of function mutants and over-expression lines are required for a detailed understanding of the role of CPKs in stress singling. Understanding the involvement of CPKs in stress signaling is particularly important because this information could be very useful in making transgenic crop species with improved abiotic and biotic stress tolerance.

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CHAPTER TWO

FUNCTIONAL CHARACTERIZATION OF CPK32

INTRODUCTION

In plants and animals, calcium plays an important role as a nutrient and as a signaling molecule. Cellular Ca^{2+} concentration changes in response to many intrinsic growth and developmental processes as well as to extracellular biotic and abiotic signals (Knight, 2000; Rudd and Franklin-Tong, 2001). Different intracellular and extracellular signals lead to dynamic patterns of spatiotemporal patterns of changes in cytosolic Ca^{2+} concentrations, called Ca signatures (McAinsh and Hetherington, 1998). A Ca signature is the specific type of change in cytosolic Ca^{2+} concentration, which can be either in the form of a spike, a wave, or a localized increase depending upon the specific type of stimulus and its magnitude. For example, the Ca signature produced during symbiosis depends upon the type of organisms involved. Mycorrhizal fungi produce Ca oscillations in the roots which are for a shorter period of time and are lower in amplitude as compared to those produced by rhizobial NOD factors (McAinsh and Pittman, 2009). Thus, each Ca signature proportionates a specific stimulus and thereby helps to coordinate an appropriate response. A particular Ca signature also depends upon the rate of stress development (Plieth et al., 1999), the previous stress exposure history (Knight et al., 1997), and the tissue type (Keigle et al., 2000).

In plants, Ca signatures are decoded by three major classes of Ca^{2+} sensors (Cheng et al., 2002). These include calmodulin (CaM) and CaM-related proteins (CMLs), which both can bind Ca^{2+}; calcineurin β-like proteins (CBL), which are similar to the β subunit of mammalian calcineurin and to neuronal Ca^{2+} sensors (Kudla, 1999; Luan et al., 2002); and, calcium dependent protein kinases (CPK), which occur primarily in plants and a few protists (Harmon et al., 2001; Harper and Harmon, 2005; Nagamune and Sibley, 2006). In Arabidopsis, there are seven genes that encode CaMs and fifty genes for CMLs. CMLs show significant structural divergence compared to CaMs, possess two to six EF hands, and differ in their substrate specificities towards CaM isoforms (MaCormack and Braam, 2003). CBLs and CBL-interacting protein kinases (CIPKs) form a large multigene family in Arabidopsis, there being at least ten CBLs and twenty five CIPKs (Kudla et al., 1999; Kim et al., 2000; Luan, 2008). Determining specific CBL-CIPK interactions have been particularly useful for understanding salt signaling mechanisms. CBLs and CIPKs such as CBL1, CIPK3, CIPK 5, and CIPK15 have been shown to be negative regulators of ABA signaling (Guo et al., 2002; Kim et al., 2003; Song et al., 2005).

CPKs occur as a large multigene family likely in all higher plant species. In Arabidopsis, there are thirty four CPKs (Arabidopsis Genome Initiative, 2000) and in rice there are thirty one CPKs. In both species, these form four phylogenetic groups depending upon sequence identities (*e.g*., Cheng et al., 2002). CPKs have four distinct domains: an N-terminal variable domain, a protein kinase domain, an autoinhibitory or junction domain, and a CaM-like domain. CPKs are a unique type of Ca^{2+} sensor since

they function both as sensor relays and as sensor responders. This dual functionality can be attributed to the presence of both a CaM-like Ca^{2+} sensor domain and a serine/threonine protein kinase responder domain in the same protein (Cheng et al., 2002). The autoinhibitory domain of CPKs acts as a pseudosubstrate and regulates Ca^{2+}-dependent activation of the kinase (Harmon et al., 1994; Harper et al., 2004). Under elevated Ca^{2+} concentrations the EF hands in the CaM-like domain bind to Ca^{2+}, which leads to the removal of autoinhibition by changing the conformation of the protein. This results in activation of the protein kinase domain, which then is able to phosphorylate a variety of protein substrates. Thus, CPKs are able to auto-activate after sensing an increase in Ca^{2+} concentration due to internal or external stimuli. The N-terminal domain is a variable region which ranges in length from 21 to 185 amino acids (Dammann et al., 2003). The N-terminal domain includes important information specifying the sub-cellular localization of particular isoforms of CPK. Most CPKs have an N-terminal Gly as amino acid number 2 and variable numbers of Cys residues at positions 3, 4 and/or 5. These sites potentially are subject to post-translational lipidation by myristoylation and palmitoylation, respectively. Myristoylation and palmitoylation are important for sub-cellular targeting of proteins in plants and animals. Apart from their membrane localization signals, these post-translational modifications are also important for shuttling proteins between membranes and the cytosol, for facilitating protein-protein interactions, and for protein stability (Nadolski and Linder, 2007; Hemsley and Grierson, 2008). CPKs have diverse sub-cellular localizations such as the plasma membrane (Schaller et al., 1992), endoplasmic reticulum (Lu and Hrabak, 2002), nucleus (Patharker and

Cushman, 2000), mitochondria (Pical et al., 1993), cytoskeleton (Putnam-Evans at al., 1989), oil bodies (Anil et al., 2000), and peroxisomes (Dammann et al., 2003). Mutations in the myristoylation and palmitoylation residue of CPKs can lead to their reduced membrane targeting and/or their increased cytosolic localization (Ellard-Ivey et al., 1999; Martin and Busconi, 2002; Lu and Hrabak, 2002).

CPKs from Arabidopsis and other plants have been implicated in many aspects of plant growth and development, hormone signaling, and responses to abiotic and biotic stresses (Cheng et al., 2002; Ludwig et al., 2003; Harper and Harmon, 2005). However, the occurrence of several isoforms in a given species can lead to isoform-specific functions or to functional redundancy (Cheng et al., 2002; Ludwig et al., 2004). Nonetheless, Urao et al. (1994) showed that two different CPKs from Arabidopsis are regulated by both salt and drought stress. Since then, a number of studies have shown that CPKs from Arabidopsis as well as other plants such as rice, tomato, ground nut, and many more, are involved in diverse aspects of abiotic and biotic stress signaling (Klimecka and Muszynska, 2007). For example, phenylalanine ammonia lyase is a key regulator of biotic stress responses to pathogenesis, which is modulated by phosphorylation by AtCPK1 (Cheng et al., 2001). There are several reports of CPKs from Arabidopsis and other species which are transcriptionally up regulated by abiotic and biotic stress stimuli. These likely also have a role in stress signaling. For example, tobacco CPKs NtCPK1, NtCPK2 and NtCPK3 are transcriptionally up regulated by fungal elicitors and osmotic stress (Yoon et al., 1999; Romeis et al., 2001). In common

ice plant CPK, McCPK1 expression is regulated by drought and salt (Patharkar and Cushman, 2000).

The expression of AtCPK3 and AtCPK6 in guard cells has been shown to regulate ABA and Ca^{2+} activation of anion channels and of ABA regulated Ca^{2+}permeable cation channels (Mori et al., 2006). These findings provide molecular genetic evidence that these two CPKs act as positive regulators of ABA signaling in controlling stomatal movements. AtCPK4 and AtCPK11 have also been shown to function as positive regulators of ABA signaling since *cpk4* and *cpk11* are ABA insensitive and also display salt hypersensitive phenotypes. CPK4 and CPK11 phosphorylate two ABA responsive transcription factors, ABF1 and ABF4 that help mediate ABA signaling (Zhu et al., 2007). In an opposite fashion, the disruption of AtCPK23 in Arabidopsis leads to enhanced salt and drought tolerance (Ma and Wu, 2007). This finding indicates that AtCPK23 acts as a negative regulator of stress signaling. In rice, OsCPK6 and OsCPK25 are up regulated by salt, drought, and cold whereas OsCPK17 is down regulated by these treatments. Also, in alfalfa, MsCPK1 is up regulated, but MsCPK2 is down regulated by cold (Monroy and Dhindsa, 1995), though specific studies on their functions are lacking. Together, the available information about CPKs suggest that numerous CPKs from a given species are differentially regulated under stress conditions and can act either as positive or negative regulators of stress signaling.

In rice, the comprehensive study of twenty nine CPKs has revealed that all the CPKs have multiple stress regulatory elements in their gene promoters (Wan et al., 2007). The same might be expected for Arabidopsis CPKs. However, Arabidopsis microarray

analyses have focused on the identification of Ca^{2+} and ABA regulated genes, showing that AtCPK32 is the only CPK among all thirty four Arabidopsis CPKs that is up-regulated by Ca^{2+} and that also possesses an ABRE coupling element (Kaplan et al., 2007). A previous study involving Arabidopsis CPK32 has shown that in yeast two hybrid assays, CPK32 phosphorylates ABF4, a transcription factor involved in ABA signaling (Choi et al., 2005). Both the protein kinase domain of CPK32 and the N-terminal variable domain are required for this phosphorylation event to occur. Choi et al. (2005) also report that CPK32 is induced by salt treatment, but not by ABA, cold, or mannitol treatments. In a separate study, CPK32 was found to be both a touch and darkness-inducible gene (Lee et al., 2004). This is consistent with results from Chotikacharoensuk et al. (2006) in which CPK32 was rapidly induced by touch, wounding, salt, and dark. This indicates that CPK32 might be an early response gene for transducing cellular responses to these stimuli. Together, the available information suggests that CPK32 is a strong candidate to have a role in ABA signaling.

To investigate function of CPK32 in depth and to understand how it is involved in ABA signaling, we have used a reverse genetics approach to characterize phenotypes with altered CPK32 protein expression. Our analysis of transgenic and mutant lines shows that the overexpression of CPK32 results in ABA and salt insensitive phenotypes, whereas disruption of the CPK32 gene results in hypersensitive phenotypes as shown by germination and early seedling growth assays. These hypersensitive phenotypes are rescued by the native CPK32 gene. Thus, we provide genetic evidence that the CPK32 protein acts as a negative regulator of ABA signaling. In addition to these phenotypes,

we report that CPK32 is transcriptionally upregulated by ABA, salt, osmotic stress, wounding, and flagellin22 peptide, and biotic stress stimuli. Finally, we report that CPK32 is a plasma membrane localized protein that mediates ABA signaling by an ABA-dependent movement from the plasma membrane to the nucleus. The post-translation lipid modifications of myristoylation and palmitoylation play a crucial role in regulating the sub-cellular localization of CPK32. Together these data indicate that CPK32 regulates a diverse array of stress related responses in Arabidopsis.

MATERIAL AND METHODS

Plant Materials and Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0; wild type, WT) were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Heterozygous seeds (T_2 generation) of one parental T-DNA insertion line of AtCPK32 (At3g57530, GK-824E02/ N479058, named *cpk32-1*) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Maize seeds (line FR922 X FR967) were purchased from Seed Genetics, Inc. (Lafayette, IN). Peas (*Pisum sativum* var. Little Marvel) were purchased from Gurneys Seed and Nursery Co. (Greendale, IN).

Arabidopsis seeds were routinely surface sterilized using 25% bleach for 5 min, stratified for 4 d at 4^{o}C, and grown on 1X MS agar plates under constant light (30 μmol m^{-2} s^{-1}) at room temperature (modified basal medium with Gamborg vitamins [PhytoTechnology Laboratories, Shawnee Mission, KS)], pH 5.7, 0.5% [w/v] sucrose, and 0.7% [w/v] phytagar [Caisson Laboratories, North Logan, UT]). Arabidopsis and pea plants also were grown in soil in a growth chamber (125 μmol m^{-2} s^{-1} with 10 h photoperiod at 23^{0}C/20^{0}C day/night). For herbicide screening, Arabidopsis plants were grown in a green house under ambient conditions. Plants were fertilized with Peter's nutrient solution once a week. Maize seeds were soaked overnight and germinated on a moist paper towel. Germinating seeds were planted in soil and grown in the dark at room temperature for 7-8 d, until bringing to the light for 12-16 h for greening.

To identify the *cpk32-1* mutant, heterozygous seeds obtained from NASC were screened for homozygosity on agar plates with 50 μg mL^{-1} sulfadiazine (Spectrum Chemicals, Gardena, CA). Out of ten lines obtained from NASC, two were homozygous. Since all these lines were obtained from one insertion event, then both homozygous lines defined the same putative mutant. The T-DNA insertion was confirmed by PCR using a left border T-DNA primer, GKLB, and a CPK32 gene specific primer, GKgCPK32 (Table 2.1). Direct sequencing showed that the insert was located in the first exon, 359 bp downstream of the start codon.

Different transgenic lines made in this study are listed in Table 2.2. These include pCPK32::GUS for expression of the CPK32 promoter by using β-glucoronidase (GUS) as a reporter; 35S::CPK32-FLAG overexpression lines in WT Col (Ox lines); the *cpk32-1* line mentioned above; and, complementation lines (Cx lines) which express the native CPK32 gene in *cpk32-1*. Further details on the number of homozygous lines obtained and the plasmid selection markers used are provided in Table 2.2.

Plasmid Constructs and Transgenic Lines

To make the pCPK32::GUS construct, a 2045 bp region immediately upstream of the start codon was amplified with long template *Taq* polymerase (Roche, Indianapolis, IN) using CPK32PRF / CPK32PRR primers. The list of all cloning primers is given in Table 2.1. The PCR product was then cloned using *Hind*III and *Xba*I restriction sites, into the binary vector pSMAB704 (Igasaki et al., 2002). The PCR product and binary

Table 2.1. *List of primers used in cloning experiments and screening for TDNA insert*

Underlined sequences are enzyme restriction sites.

Primer Name	**Primer Sequence**
ABF4F	5'-CGC GGA TCC ATG GGA ACT CAC ATC AAT TTC-3'
ABF4R	5'-AGG CCT CCA TGG TCC GGT TAA TGT CCT-3'
CC45AAR	5'-TCC TGC TGT TCC GGC TGC ATT ACC CAT GGA TCC GAT GGG TGC-3'
C4AC5AF	5'-GCA CCC ATC GGA TCC ATG GGT AAT GCA GCC GGA ACA GCA GGA-3'
CPK32F	5'-CGG GAT CCA TGG GTA ATT GTT GCG GAA CA- 3'
CPK32R	5'- AAG GCC TTC TTG TAT CAC CAT TGA CCT GCA-3'
CPK32PRF	5'-ACC CCA AGC TTC ATC TGC TGA GAC TGA TAC GA-3'
CPK32PRR	5'-GCT CTA GAG ACT TTT CCG ATC AAA CCC AA-3'
gCPK32F	5'-GCT CTA GAC ATC TGC TGA GAC TGA TAC GAC TA-3'
gCPK32R	5'-TCC CGG GCC CGT CGA CCA CCA GGA CAA AAC G-3'
g32CPK32FF	5'-GTC AAT GGT GAT ACA AGA CTG ATC TTC CTG CTG CTA CTG TTT TGA GAG AGG CTT AAT CTG-3'
g32CPK32FR	5'-CAG ATT AAG CCT CTC ACT AAA CAG TAG CAG CAG AAG CAT CAG TCT TGT CTA CCA ATT GAC-3'
GKLB	5'-ATA TTG ACC ATC ATA CTC ATT GC
GKgCPK32	5'-CCT TTA AAG CAA TGC TAC AAG TC-3'
G2AF	(5' -CGT GCA CCC ATC GGA TCC ATG GCA AAT TGT TGC-3'
G2AR	5'-GCA ACA ATT TGC CAT GGA TCC GAT GGG TGC ACG-3'
G2C4C5F	5′-GCA CCC ATC GGA TCC ATG GCA AAT GCA GCC GGA ACA GCA GGA- 3'

G2C4C5R	5′-TCC TGC TGT TCC GGC TGC ATT TGC CAT GGA TCC GAT GGG TGC- 3'
PBP1F	5′-CGG AAT CCA TGG CCC AAA AGG TGG AAG-3′
PBP1R	5′- AAG GCC TGT TGG ATA AAG GAC GAA CA-3′

Table 2.2. *List of mutant and transgenic lines made for this study*

WT = wild type.

Transgenic Line (notes)	Vector Used	Cloning Sites	Selection Marker	Homozygous Line Numbers
pCPK32::GUS in Col WT (~2 kb genomic DNA)	pSMAB704	*Hind*III / *Xba*I	Bar herbicide (glufosinate)	3
35S::CPK32-FLAG in Col WT (coding sequence)	pCB302-ES	*BamH*I/ *Stu*I	Bar herbicide	4
cpk32-1 in Col WT (T-DNA insertion mutant)	T-DNA	-	Sulfadiazine	1
pCPK32::CPK32-FLAG in *cpk32-1* (genomic DNA)	pC1301	*Xba*I / *Sma*I	Hygromycin	4

vector were electrophoresed on a 1.5% [w/v] Nusieve agarose gel (Lonza, Rockland, ME), then ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA) by an in-gel reaction done overnight at room temperature. Positive clones from transformed *E. coli* (MC1061) were confirmed by diagnostic restriction enzyme digests and by direct sequencing of plasmid DNA.

To make the CPK32 overexpression construct, CPK32 cDNA was amplified from a library made from 7-d-old Arabidopsis (Col) seedlings, using Phusion DNA Polymerase (New England Biolabs, Ipswich, MA) and CPK32F / CPK32R primers. The amplified product was cloned as a C-terminal fusion gene with either green fluorescent protein (GFP) or FLAG tag in the HBT vector (Kovtun et al., 1998), using *Bam*HI and *Stu*I cloning sites. The ligations and confirmation of clones were done as described above. For generating CPK32 overexpression lines, CPK32-FLAG was sub-cloned in to the binary vector pCB302ES (Xiang et al., 1999) using *Bam*HI and *Pst*I cloning sites. This binary vector was then transformed into plants through Agrobacterium-mediated transformation, as described below.

The *cpk32-1* mutant plants were transformed using a 5.4 kb genomic fragment which contained the full length CPK32 gene, including the promoter and 5′ UTR (2045 bp), the 3′ UTR (343 bp), and the 500 bp putative terminator region. Genomic DNA was isolated from leaves of WT plants using the DNeasy plant mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. CPK32 gene was amplified using gCPK32F / gCPK32R primers with Phusion DNA polymerase (New England Biolabs). This amplified product was then cloned into pT7T3 vector using *Xba*I and *Sma*I

restriction sites. The pT7T3 vector DNA was a generous gift from Dr. William Marcotte, Jr. (Clemson University). Positive clones were confirmed with diagnostic restriction enzyme digests and also by sequencing. A FLAG epitope tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was then introduced before the stop codon of CPK32 by site-directed mutagenesis, using the QuikChange mutagenesis procedure with Pfu Turbo polymerase (Stratagene, La Jolla, CA). The primers used for this were gCPK32FF / gCPK32FR (Table 2.2). This construct also was completely sequenced to confirm the proper insertion of the FLAG tag and to verify that no frame shift or other mutations occurred during the mutagenesis reaction. This construct was then sub-cloned into the pC3101 binary vector with *Xba*I and *Sma*I cloning sites. The pC1301 vector (Cambia, Canberra, Australia) was a gift from Dr. Julia Frugoli (Clemson University). This construct was then introduced into Agrobacterium for plant transformation.

To make the different transgenic lines, binary constructs were introduced into *Agrobacterium tumefaciens* (GV3101) by electroporation and were transformed into Arabidopsis using the floral dip method (Clough and Bent, 1998). For pCPK32::GUS, three independent homozygous lines were selected in the T_3 generation from eight T_2 lines segregating 3:1 for *bar* herbicide resistance (200 μM glufosinate ammonium, Rely 200, Bayer Crop Science, Kansas City, MO). For the 35S::CPK32-FLAG overexpression (Ox) lines, four independent, homozygous lines were selected in the T_3 generation from twelve T_2 lines which showed 3:1 segregation for herbicide resistance. To identify *cpk32-1* complementation (Cx) lines, Agrobacterium transformed seeds (T_0) were selected on agar plates using hygromycin (20 μg mL^{-1}). Thirty six T_1 lines were selected.

From these, a total of fourteen lines showed 3:1 segregation and four independent homozygous lines were obtained.

Site Directed Mutants of CPK32 and Other Constructs

Site directed mutants of CPK32 were generated using Stratagene QuikChange mutagenesis (Stratagene, LaJolla, CA). A myristoylation mutant (G2A), a palmitoylation double dmutant (C4A-C5A), and a myristoylation-palmitoylation triple mutant (G2A-C4A-C5A) were made using mutation specific primer pairs listed in Table 2.1. Both CPK32-FLAG and CPK32-GFP constructs were used to create the corresponding mutant constructs with either GFP or FLAG tag. Other constructs such as AtABF4 (At3g19290) and AtPBP1 (At3g16260) were cloned following same cloning procedure as described for CPK32. AtABF4 was cloned into the HBT vector with C-terminal GFP tag and AtPBP1 was cloned with a C-terminal double hemagglutinin (HA) tag.

Histochemical GUS Staining and GUS Activity Assay

Homozygous transgenic plants expressing pCPK32-GUS were used for histochemical GUS staining, as described by Crone et al. (2001). Staining was done on seedlings, as well as on a variety of plant tissues including full sized leaves, stems of three week-old plants, whole flowers, floral buds, anthers from open flowers prior to dehiscence, mature siliques, and mature seeds. Samples were incubated in GUS staining

buffer (100 mM K-phosphate-KOH, pH 7.0, 100 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.06% [v/v] Triton-X 100, and 0.5 mg mL^{-1} of X-Gluc (Gold Biotechnology, St. Louis, MO) for up to 2 h at 37^0C. Due to the high GUS activity in seedling roots, pollen, and embryos, staining was performed in these cases for only 15 min. For staining embryos, seeds were hydrated on MS plates for 10-15 min, the seed coat was removed using forceps, and GUS staining solution was added to embryos. After staining, the tissues were washed with 70% ethanol and then de-stained with 95% ethanol for 12-16 h. Images of GUS-stained seedlings and tissues were taken using a stereomicroscope (Nikon SMZ1500), with a MicroPublisher CCD cooled color camera, and Image Pro Plus v 5.0 software.

In one series of experiments, the regulation of seedling GUS activity by abiotic and biotic stress stimuli was examined. Excised leaves from homozygous plants expressing pCPK32-GUS were placed in water alone for 2 h or with 10 μM ABA, 100 mM NaCl, 10% [w/v] PEG, 100 μM acetosyringone, 1 mM salicylic acid (Mahalingam et al., 2003), 1 μM flagellin peptide (Flg 22; Molinier et al., 2006), or 100 μM methyl jasmonate. For cold and heat treatments, excised leaves were kept in water for 2 h at 4^0C and 40^0C, respectively. For the wounding treatment, 3-4 mm cuts were made on leaf lamina with a sharp blade, without cutting the midrib, as described by Raymond et al. (2000). For the dehydration treatment, leaves were excised and allowed to dry on the bench top on a moist kimwipe paper for ~2 h (to 75% of initial fresh weight), as described by Zhu et al. (2007).

Extractable leaf GUS enzyme activity was measured as described by Jefferson et al. (1987) and Tian et al. (2002). The seedling or leaf tissue was ground in extraction buffer containing 50 mM NaH_2PO_4-NaOH (pH 7.0), 10 mM EDTA, 0.1% [v/v] Triton X-100, 0.1% [w/v] sodium L-lauryl sarcosine, and 10 mM β-mercaptoethanol. The fluorogenic reaction was performed using 10 µl of extract and 30 µl of extraction buffer with 1 mM 4-methyl umbelliferyl glucuronide (MUG, Sigma-Aldrich, St. Louis, MO) at 37^{o}C for 2 h. The reaction was stopped with 360 µl of 0.2 M Na_2CO_3. Fluorescence was measured in a 96-well microtiter plate format using a GENios spectrophotometer (Phoenix Research Products, courtesy of Dr. Sarah Harcum, Clemson University) at 360 nm excitation and 465 nm emission wavelengths. Product formation was determined from standard curves made using 0.1-1 µM 4-methyl-umbelliferone (Sigma-Aldrich). Sample GUS activities were normalized to protein content from each extract determined by Coomassie dye binding assay (Bio-Rad, Hercules, CA).

Stress Response Assays

Seeds of all genotypes were grown in soil, then harvested and dried at the same time in order to minimize age-dependent variation associated with seed dormancy. All seeds were stored at 4^{0}C until they were used for assays. Seeds were surface sterilized and stratified as described earlier. For salt and ABA response assays, 25 seeds from each genotype (Col-WT, *cpk32-1*, Ox-Ln9, and Cx-Ln29) were placed on 1X MS plates containing 0-125 mM NaCl or 0-0.8 µM ABA (Sigma-Aldrich). Polyethylene glycol

(PEG) infused agar plates were used to induce osmotic stress. For this treatment, 1X MS plates were soaked for 14 h with an equal volume (20 ml) of different MS media containing 125, 250, 400, and 550 g L^{-1} PEG 8000 (Fluka, Sigma-Aldrich). These treatments are reported to induce a final water potential of -0.25, -0.5, -0.7, and -1.2 MPa, respectively, as described by Weele et al. (2000). For germination assays, seeds were scored for radical protrusion ($\geq$ 2-3 mm) at 24 h intervals starting at 48 h from plating. Seedling growth assays were done during early development, before the appearance of true leaves and were documented using a digital camera. All phenotype assays were carried out on triplicate plates, with at least two biological replicates. For dehydration studies, plants were grown with regular watering for 3 wks and then withheld from watering for 2 wks. The plants were scored for wilting after 9 days of water withholding. Plants were re-watered for after 15 days. Plants were counted for survival 10 days after re-watering, for continued growth and completion of life cycle. In the water loss assay, we used detached leaves from each genotype and dehydrated them on the bench top on a moist kimwipe for up to 5 h. The fresh weights were measure using an analytical balance every 1h interval.

Protoplast Transient Expression Assays

Depending on particular applications, mesophyll protoplasts were isolated from leaves of Arabidopsis (3-wk-old), maize (7-8-d-old) and pea (3-wk-old) plants. Isolated protoplasts were transfected using PEG 4000 (Fluka; Hwang and Sheen, 2001; Yoo et al.,

2007). Transfection efficiencies were routinely >70% for maize and peas, and about 40% for Arabidopsis, as evaluated using CPK32-GFP or ABF4-GFP. Following transfections, protoplasts were incubated for 8-10 h at low light, collected by low speed centrifugation, and used for different assays. For sub-cellular localization, protoplasts were visualized using an LSM 510 confocal laser-scanning microscope (Axiovert 200 M fluorescence microscope, Carl Zeiss, Dublin, CA) and a 40X oil objective. Nuclei were directly stained with Hoechst dye (Sigma-Aldrich) and visualized using an Axiovert 200 M fluorescence microscope with Apotome (Carl Zeiss) and appropriate band-pass filter (Chroma # 3100, Chroma Technology Corp., Rockingham, VT). In one experiment, protoplasts expressing CPK32-GFP for 8 h were treated with 5 and 10 μM ABA (Sigma-Aldrich) and examined for 15-20 min thereafter.

Production of CPK32 Antibody and Immunoblotting

Anti-rabbit polyclonal CPK32 antibody was raised against a 20 residue peptide made from the N-terminal variable region of AtCPK32, ^{22}RKKQNPFSIDYGLHHGGGD^{40} (YenZym, LLC, South San Francisco, CA). The antibody was affinity purified to bound peptide prior to use. The selected sequence has minimal similarity to other AtCPKs (Fig. 2.6). The specificity of the anti-CPK32 antibody was determined from Western blots using different AtCPKs expressed in protoplasts and using *cpk32-1* seedling extracts. Available clones of AtCPK10 (At1g18890), AtCPK11 (At1g35670), AtCPK13 (At3g51850), AtCPK30 (At1g74740)

and cotton GhCPK1 were expressed in maize protoplasts as either FLAG or GFP fusion proteins, with CPK32-FLAG and CPK32-GFP clones as positive controls. Expression of the different CPKs was first established by probing the blots with anti-FLAG M2 antibody (1:5000, Sigma-Aldrich) or by visualizing GFP tagged proteins using fluorescence microscopy. Western blots were stripped and reprobed with anti-CPK32 antibody (1:2500). Anti-rabbit HRP secondary antibody (1:5000) was used against anti-CPK32 antibody and anti-mouse HRP secondary antibody (1:7500) was used against anti-FLAG M2 antibody (Pierce Biotechnology, Rockford, IL). Anti-actin primary antibody was used as a loading control (Dr. Richard Meager, University of Georgia, Athens, GA).

For protein sample preparation, seedlings were extracted in plant extraction buffer containing 50 mM HEPES-KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 15 mM KCl, 10% [v/v] glycerol, 0.1% [v/v]Triton X-100, and 1X protease inhibitors ('complete', Roche Molecular Biochemicals, Indianapolis, IN). Protein contents were measured and 2 μg were separated on 10% SDS-PAGE gels. For experiments using expressed CPKs in protoplasts, the pelleted protoplasts were directly lysed and denatured at 95^0C in 2X SDS loading dye. The proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA) using a semi-dry transblotter (Bio-Rad). Membranes were blocked in 5% non fat dry milk, and then incubated with appropriate antibodies as described above. The chemiluminescence reaction was performed using SuperSignal West Pico reagents (Pierce Biotechnology) and detected with X-ray film (Blue X-ray, Phenix Research Products, Candler, NC).

Protein Labeling and Immunoprecipitation Assays

Protein labeling and co-immunoprecipitation assays were carried out generally as described by Balasubramanian et al. (2007). Maize protoplasts were co-transfected with 8 μg of cesium chloride-purified plasmid DNA constructs containing WT CPK32-FLAG or site-directed mutant constructs (G2A, C4A-C5A, and G2A-C4A-C5A). All isotopes were purchased from Perkin-Elmer (Waltham, MA): [^{35}S]-Met (Express Labeling Mix; 1175 Ci mmol^{-1}), [^{3}H]-myristic acid (9,10-3H[N]-tetradecanoic acid; 30 Ci mmol^{-1}), and [^{3}H]-palmitic acid (9,10-3H[N]-palmitic acid; 30 Ci mmol^{-1}). The [^{3}H]-myristic acid and [^{3}H]-palmatic acid were dried under liquid N_2 gas and then resuspended in 100 μl of protoplast incubation medium before adding to protoplasts. Isotopes were added after 90 min of dark incubation of transfected protoplasts, using 25 μCi of [^{35}S]-Met, 500 μCi of [^{3}H]-myristic acid, or 500 μCi of [^{3}H]-palmitic acid. Protoplasts were harvested after 10 h and subsequent pellets were resuspended in 200 μl of immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 1% [v/v] Triton X-100, 1 mM dithiothreitol, 1 mM NaF, 1 mM $NaVO_3$, 1X protease inhibitors, and 0.05% [v/v] SDS). Lysed protoplasts were incubated for 2 h at 4^0 C with anti-FLAG M2 antibody. Antibody-bound proteins were captured (1 h, 4^0 C) on protein A agarose beads (Roche, Indianapolis, IN) then eluted in 2X SDS buffer, heat treated, and electrophoresed on a 10% SDS-PAGE gel. For fluorography, gels were treated with 'Enhance' (Perkin-Elmer)

prior to drying. Dried gels were exposed to X-ray film at -80^{0}C for up to 4 d for [^{35}S] and 5-7 wks for [^{3}H].

CPK32-FLAG Pull Down and Protein Interaction Analysis

Seeds of Col WT, Ox-Ln9, and *cpk32-1* were grown vertically on 1X MS plates as described earlier. For pull down experiments, 500 mg of seedling tissue from each genotype were harvested and immediately frozen in liquid nitrogen. Tissues were ground using a mortar and pestle with 2 ml of extraction buffer containing 100 mM HEPES-KOH pH 7.5, 5 mM $MgCl_2$, 15 mM KCl, 0.5% [v/v] CHAPS or glucoside detergent (dodecyl maltoside), 1 mM EDTA, 2 mM dithiothreitol, 10% [v/v] glycerol, and 1X protease inhibitors. Extracts were centrifuged at 12,000 g for 10 min at 4^{0}C, soluble protein contents were measured, and supernatants were kept on ice. In one experiment, 50 μg of proteins each from Col, Ox-Ln9, and *cpk32-1* were separated by Blue Native gel electrophoresis (Swamy et al., 2006). The gels were made in buffer containing 150 mM Bis-Tris (pH 7.0) and 200 mM ε-aminocaproic acid. Proteins were separated on 4-15% non-denaturing polyacrylamide gels at 4^{0}C at 100 V for 1 h, then at 300 V for 3 h using separate anode (50 mM Bis-Tris pH 7.0) and cathode buffers (15 mM Bis-Tris pH 7.0, 50 mM Tricine, and 0.02% [w/v] Coomassie blue G250). The gels were then transferred to Immobilon-P membrane (Millipore) using a semi-dry transblotter, with immunodetection carried out as described earlier.

Co-immunoprecipitation assays were done with 900 µg of total protein. Extracts were incubated with 4 µl of anti-CPK32 polyclonal antibody for 2 h at 4^0C, then with 10 µl protein A agarose beads for 1 h at 4^0C. The Protein A agarose beads were collected by 15 s centrifugation (maximum microfuge speed). Beads were washed 3 times with 250 µl of buffer (50 mM Tris-HCl pH 7.8, 150 mm KCl, 10% [v/v] glycerol), re-suspended in 2X SDS sample buffer, and proteins were separated on 10% SDS PAGE gels. To visualize proteins bands, the gels were stained with Colloidal Coomassie (Invitrogen, Carlsbad, CA). One of the protein bands from the *cpk32-1* extract was excised for further analysis by mass spectrometry (the University of Nevada Proteomics facility, IDeA Network of Biomedical Research Excellence, INBRE, Nevada, www.unr.edu/inbre).

RT-PCR Analysis

To examine organ-specific expression of CPK32 mRNA, different samples were collected from Col WT plants. For whole plant samples, 7-d-old seedlings were collected from MS plates. Roots from 10-d-old seedlings were excised at the root-hypocotyl junction. Young leaves and mature leaves were harvested from 3-wk-old chamber grown plants. Inflorescences and siliques were collected from flowering plants. Germinating seeds were harvested after 60 h on MS plates, at which time the radicals protruded out of seeds.

For RNA extraction, all tissues were quickly frozen in liquid nitrogen immediately after harvesting. Total RNA was extracted from 100 mg of tissue using the

RNeasy plant mini kit (Qiagen), according to the manufacturer's instructions. One μg of total RNA was converted to cDNA using the Protoscript II RT-PCR kit (New England Biolabs). The cDNA was diluted ten-fold and PCR reactions were performed using 2 X PCR master mix (New England Biolabs). Template amounts were titrated using Arabidopsis ubiquitin5 (UBQ5) as a control along with gene specific amplification, by varying the cycle number to avoid product saturation. CPK32 was amplified using CPK32RTF and CPK32RTR primers (Table 2.3).

Quantitative Real Time PCR Analysis

The response of CPK32 transcript levels to different abiotic and biotic stress signals was analyzed using Real Time PCR analysis. Seven-d-old seedlings were grown vertically on plates before treatments. Seedlings were transferred for 2 h to solutions containing water, ABA (10 μM), NaCl (100 mM), PEG (20%, [w/v]), acetosyringone (100 μM), salicylic acid (1 mM), flagellin peptide (Flg 22, 1 μM), and methyl jasmonate (100 μM). Also, seedlings in water were cold (4^0C) or heat (40^0C) treated for 2 h. Dehydration was done as described earlier for the GUS activity assay. For wounding, small cuts were made on the leaves of seedlings with a sharp blade. Total RNA was extracted as described above. Fifteen μg of RNA was treated with RQ10 DNAse (Promega, Madison, WI), precipitated using phenol-chloroform-isoamyl alcohol (25:24:1), and re-suspended in RNase free water. One μg of RNA was used for cDNA synthesis as described earlier. Template cDNA was amplified using aniCycler with iQ

SYBR Green Super Mix (Bio-Rad Laboratories), with the CPK32RTF/R primers and the UBQ5F/R primers as an internal control. PCR cycle conditions were as follows: 95 ^{0}C for 3 min, then 40 cycles of 95 ^{0}C for 30 sec, 55 ^{0}C for 10 sec, and 72 ^{0}C for 20 sec. A melting curve was performed at the end of the Real Time PCR reaction, starting at 55^{0}C with 0.5^{0}C increments up to 95^{0}C. PCR cycle threshold values (Ct) were determined from PCR baseline subtraction curve fit data using default parameters of the iCycler software package. The efficiency (E) of PCR reaction was calculated by performing a 5-fold dilution series (1:5, 1:25 and 1:125) for each primer pair. The slope of the regression

Table 2.3 *List of primers used in real time and in semi-quantitative PCR reactions*

Name	Sequence
UBQ5 F	5'-GTG GTG CTA AGA AGA GGA AGA-3'
UBQ5 R	5'-TCA AGC TTC AAC TCC TTC TTT-3'
CPK32 F	5'-AGC TGA GGA CAG CTG TTG AT-3'
CPK32 R	5'-AGG TTT CAG GTC CCT GTG CAT-3'
PBP1 F	5'-CAA GCC CAA GAT GGA ATC TCA GC-3'
PBP1 R	5'-TGT GGC CTT CCT CTT TGA GTT CG-3'
RD 22 F	5'-GTT CGC GGG AAA GAA ATG AAT GTC C-3'
RD 22 R	5'-CCG CGA TTT TGT ACT TCT GCA TCG-3'
RD29A F	5'-AGA CCC CGA TAA CGT TGG AGG AA-3'
RD29A R	5'-CGG CGA ATC CTT ACC GAG AAC AG-3'
COR47 F	5'-GCG ACG ACA ACA GAG GTT ACG G-3'
COR47 R	5'-TCT GCT GTC TTG TCG TGG TGT CC-3'
RAB 18 F	5'-GAG CAA CTC CAC AAG GAA AGT GGT G-3'
RAB 18 R	5'-CCG AAT GCG ACT GCG TTA CAA AC-3'
CBL1 F	5'-CTT GCC ATC ACC GTT CAC CAA TCT T-3'
CBL1 R	5'-CGT GTC CTC GAA ATT CTT TTG CTG-3'
NCED3 F	5'-TCG TAC CTG ACC AGC AAG TCG TTT-3'
NCED3 R	5'-AGA TGA TCG GAC GGC GAG TTG ATT-3'
MYC2 F	5'-ACG CTA TAT TCT GGC AAC CGT CGT-3'
MYC2 R	5'-TCA CAA CCC GAC CCG GAT AAT TGA-3'
DREB2A F	5'-TGG CTC GTC TTA ATT TCC CTC GGT-3'
DREB 2A R	5'-CCT GCT GTT GTT GCT GAC AGG TTT-3'

line from a plot of cycle threshold (Ct, X axis) versus the log of the starting template concentration (Y axis) was used in the equation $E = 10^{(1/slope)}-1$. Three independent biological replicates were performed, with each replicate having three experimental replicates. Ct values for the CPK32 amplification reaction were normalized to UBQ5 amplification for each reaction.

RESULTS

Organ and Tissue Expression of CPK32

In this study, we took several different approaches to better understand the function of AtCPK32. Since the expression of a protein can provide insight about its function, we first wanted to identify where in the plant CPK32 is expressed. Microarray analyses of Arabidopsis has revealed that CPKs, including CPK32, are expressed in almost all plant organs. To directly examine the organ specific expression of CPK32, we used two separate approaches. First, we used semi-quantitative RT-PCR to analyze the relative abundance of CPK32 mRNA in various plant organs. Second, we examined expression of the CPK32 promoter (pCPK32) in a transgenic line that we made which expresses the pCPK32-GUS fusion construct.

We looked at the transcript abundance of CPK32 in young leaf, mature leaf, inflorescence, silique, root, and germinating seeds. While confirming that CPK32 transcript occurs in all of these plant organs (Fig. 2.1), we found that CPK32 transcript was relatively more abundant in roots, inflorescence, and germinating seeds, and least abundant in siliques. We also examined GUS expression in pCPK32-GUS transgenic lines after histochemical staining. In young seedlings, GUS expression was observed in roots, hypocotyls, and cotyledons (Fig. 2.2A). In roots, GUS expression was mainly confined to the root cortex and was not found in the root vasculature. Interestingly, GUS staining was most rapid (within 15 min) and most prominent in the root cap, occurring

both in the root columella as well as in the lateral root cap cells (Fig. 2.2B). In the aerial plant parts, GUS staining was observed in the hydathodes of fully expanded

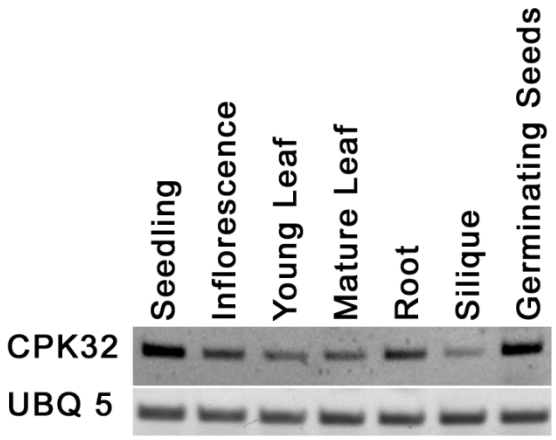

Figure 2.1. Organ specific expression of CPK32 in Arabidopsis plants as revealed by RT-PCR analysis. The template amounts were normalized using ubiquitin5 (UBQ5) as an amplification control. Product size for CPK32 is 292 bp and for UBQ5 is 254 bp.

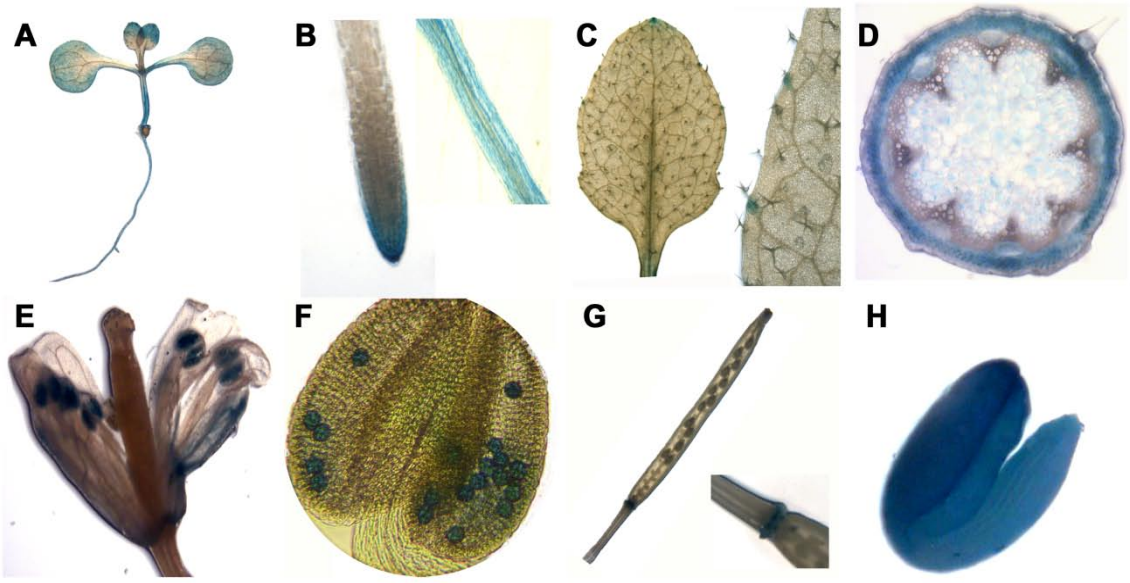

Figure 2.2. Organ specific expression of pCPK32 in pCPK32-GUS plants. Different organs from were incubated in GUS staining solution for different times as described in Materials and Methods. A, Whole 7-d-old seedling. B, Root from 7-d-old seedling. The inset shows staining of the root cortex. C, Leaf from 3- wk-old soil grown plant. D, Stem cross section of 4-wk-old plant. E, Flower. F, Anther before dehiscence. G, Mature green silique. H, Embryo.

leaves, but not in the lamina (Fig. 2.2C). In stems, GUS staining was restricted to the cortex and the phloem cells of vascular bundles as shown in cross section (Fig. 2.2D). In flowers, staining was restricted to the anthers, with the highest staining in pollen (Fig. 2.2E, F). Similar to GUS staining in the root cap, pollen was also stained very rapidly (within 10 min). Other floral parts, as well as immature buds, did not show any staining. GUS expression was observed in siliques, but the staining was restricted to the abscission zone of the mature siliques (Fig. 2.2G). The embryos also showed relatively high pCPK32 activity, as shown by the extensive GUS staining (Fig. 2.2H). Thus, the results of RT-PCR and pCPK32-GUS expression indicated that CPK32 is expressed in all plant organs, but not in all plant cells. Equivalent results were observed upon staining of organs from an independent transgenic line (data not shown).

ABA and Stress Regulatory Elements in the CPK32 Promoter

We next examined the sequence of the CPK32 promoter for possible *cis* acting regulatory elements, using publicly available resources. We identified numerous ABA and stress related response elements (Table 2.4 and 2.5) using both AGRIS (The Arabidopsis Gene Regulatory Information Server; Pilaniswamy et al., 2006), and PLACE (Plant *cis*-acting regulatory DNA elements [PLACE; Higo et al., 1999]). With AGRIS, there were twenty one predicted response elements for ABA and stress regulation of the CPK32 promoter (Table 2.4). These include a DRE element for drought resistance, a MYC2B element for drought and osmotic stress regulation, and several WRKY-box

elements involved in wounding, pathogens and defense responses, which could influence expression of the CPK32 promoter. Using PLACE, we found many additional predicted *cis*-acting elements in the CPK32 promoter (Table 2.5). These include twenty nine ABA

Table 2.4. *ABA and stress responsive binding sites found in the CPK32 promoter using AGRIS (http://arabidopsis.med.ohio-state.edu/)*

BS Name	Binding Site Sequence	Binding Site Family/TF	No. of Sites
W-box promoter motif	ttgact	WRKY	6
DPBF1&2 binding site motif	acacaag	bZIP	5
MYB4 binding site motif	aacaaac	MYB	4
HSEs binding site motif	agaagattct	HSF	2
DRE-like promoter motif	tgccgacaa	...	1
AtMYC2 BS in RD22	cacatg	BHLH	1
ATHB6 binding site motif	caattatta	HB	1
ATB2/AtbZIP53/AtbZIP44/GBF5 binding sites in genes responsive to hypo-osmolarity	actcat	bZIP	1

response elements, including three ABA-response elements (ABRE) and ABRE coupling elements (ABRE-CE). Also predicted are twenty three elements related to disease, pathogenesis, defense, and wounding. These include a number of W-box motifs for binding WRKY transcription factors and five DPBF1&2 binding site motifs, which represent ABA responsive and embryo specification elements for binding bZIP-type transcription factors. There also occur four MYB4 transcription factor binding sites,

which are responsible for regulating genes involved in environmental stresses, and one DRE like element, which is thought to bind a stress-inducible transcription factor involved in drought, salt, and freezing. Thus, the abundance of ABA and multiple stress response elements in the CPK32 promoter indicated that CPK32 might be regulated by a variety of abiotic and biotic stress stimuli, some of which involve ABA.

Table 2.5. *ABA and stress response binding sites found in the CPK32 promoter using PLACE (http://www.dna.affrc.go.jp/PLACE*. The number of binding sites show total number of DNA binding sites)

Name of Binding Sites	No. of Binding Sites
ABA response motifs	29
Disease, pathogenesis, defense and wounding	23
Dehydration Motifs	12
DPBF 1&2 binding sites	5
MYB4 binding sites	4
DRE like promoter motif	1

The Gene Expression of CPK32 Responds to Stress Signals

The presence of so many predicted stress response elements in pCPK32 indicated that CPK32 gene expression is regulated by multiple diverse stresses. To test this possibility, we examined the influence of stress stimuli on pCPK32-GUS activity and on CPK32 transcript abundance. To measure CPK32 promoter activity, we used both histochemical GUS staining (Appendix A.1) and a direct GUS enzyme activity assay.

For the GUS enzyme assay, seedlings expressing pCPK32-GUS were grown vertically on MS plates for 7 d, then removed and treated with ABA, salt, dehydration, PEG, heat or cold to produce, or simulate, abiotic stresses. Seedlings were also treated with salicylic acid, methyl jasmonate, Flagellin 22, and wounding in order to produce, or simulate, biotic stresses. Most of these stress stimuli induced the CPK32 promoter activity as measured by the GUS activity of treated whole seedlings (data not shown). However, since the CPK32 promoter activity is already very high in roots, and to some extent in cotyledons, this seedling based assay was unsuitable due to the rather high background GUS activity. Therefore, we instead treated leaves of young plants, which have lower endogenous promoter activity (see Fig. 2.2C, Appendix A). This allowed us to readily quantify even small changes in GUS activity. By this approach, the expression of pCPK32-GUS activity was induced by many stress agents about two-fold, including ABA, salt, and osmotic stresses, but not dehydration, heat and cold treatments (Fig. 2.3). Biotic stresses including Flagellin 22 peptide, acetosyringone, and wounding also resulted in two-fold increased activity. However, treatments with salicylic acid and methyl jasmonate had little effect on pCPK32-GUS activity.

In a complementary experiment, we tested the influence of these same abiotic and biotic stress treatments on the transcript abundance of CPK32. For this, we grew and treated as above 7-d-old WT seedlings and then measured CPK32 transcript levels using quantitative PCR (qPCR). The qPCR analysis is a very sensitive technique to detect changes in CPK32 transcript levels. When compared with the control treatment,

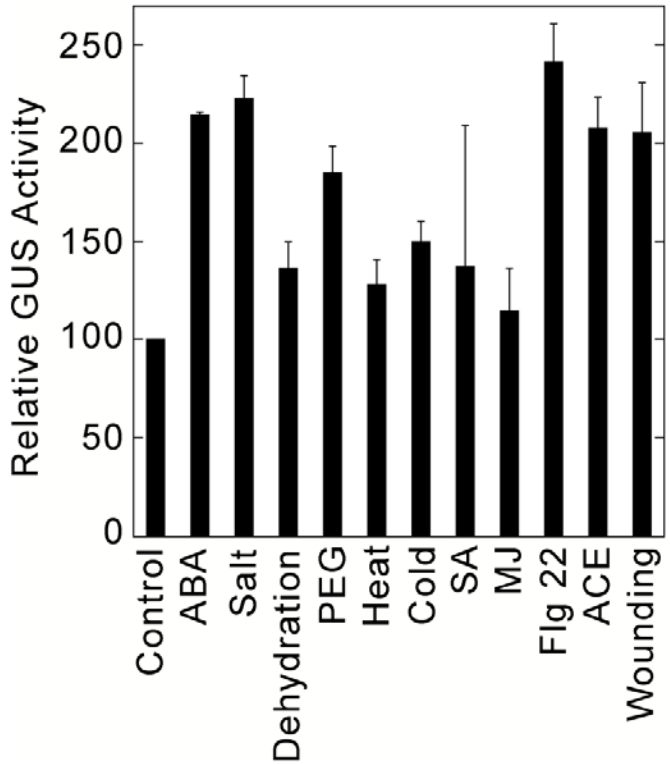

Figure 2.3. Regulation of pCPK32-GUS activity by abiotic and biotic stress stimuli. The graph shows changes in relative activity pCPK32 are shown from treated leaves. GUS activities were normalized to protein content from each extract. The control leaves were treated with water at room temperature. The following 2 h stress treatments were used: ABA = 10 μM; salt = 100 mM NaCl; PEG = 10%; heat = 40^0C; cold = 4^0C; SA = 1 mM salicylic acid; MJ = 100 μM methyl jasmonate; Flg 22 = 1 μM bacterial flagellum peptide; and, ACE = 100 μM acetosyringone. Dehydration was performed by keeping leaves on moist kimwipes to 75% of initial fresh weight. Wounding was performed by making small cuts on leaf lamina without damaging the midrib, as described in Materials and Methods. Values are means of 3 measurements ± 1 SD.

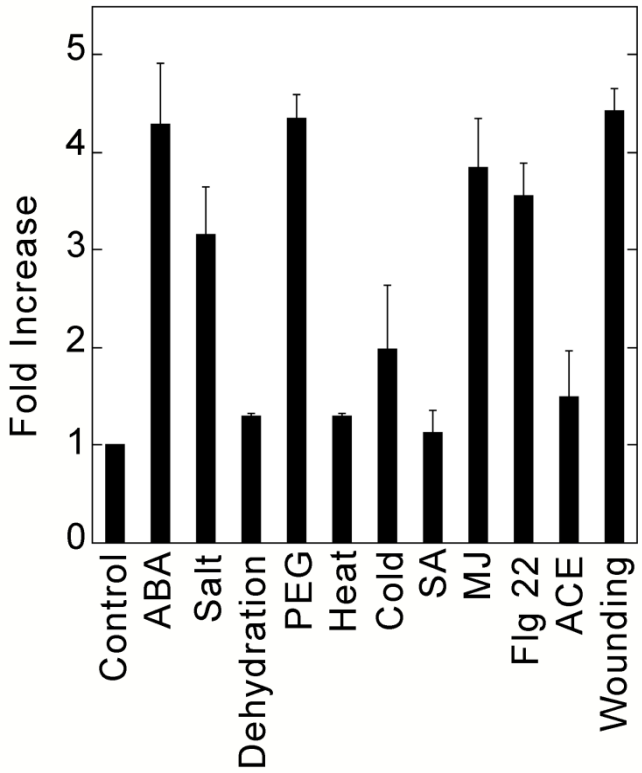

Figure 2.4. Real-Time PCR analysis of CPK32 transcript regulation by abiotic and biotic stress stimuli. The amount of transcript induction is given as fold increase relative to the control treatment with water alone. Seedlings were treated with stress stimuli as described in Fig. 2.3 and in Materials and Methods.

corresponding CPK32 transcript levels were 3-4-fold more abundant following treatments with ABA, salt, PEG, methyl jasmonate, Flagellin 22 peptide, and by wounding (Fig. 2.4). Furthermore, differential responses to abiotic and biotic stresses were much better resolved using qPCR. CPK32 transcript levels were least influenced by dehydration, heat, salicylic acid, and acetosyringone (≤ 50%). Together the results of the GUS activity assay and qPCR measurements indicate that CPK32 is a stress responsive gene and that it is induced by diverse abiotic and biotic stimuli.

Identification of CPK32 Mutant Plants and Validation of CPK32 Transgenic Lines

We used a reverse genetics approach to characterize phenotypes associated with the altered expression of CPK32. Seeds of T-DNA insertion lines in CPK32 (GK-824E02) in Col background were obtained from the NASC seed stock center and were designated as *cpk32-1*. The homozygous lines were obtained by screening heterozygous seeds using sulfadiazin selection. Using genomic DNA with a T-DNA left border specific primer and a gene specific primer (Table 2.1), the insertion was mapped to the first exon of the CPK32 gene, 359 bp downstream of the start codon (Fig. 2.5A). Using semi-quantitative RT-PCR and CPK32 specific primers, we did not find detectable transcript for CPK32 in the *cpk32-1* seedlings, but we could readily amplify the product from WT seedlings (Fig. 2.5D).

We made CPK32 overexpression lines (Ox) in Col background, using CPK32 cloned as a fusion construct with a C-terminal FLAG tag and a 35S promoter (Fig. 2.5B).

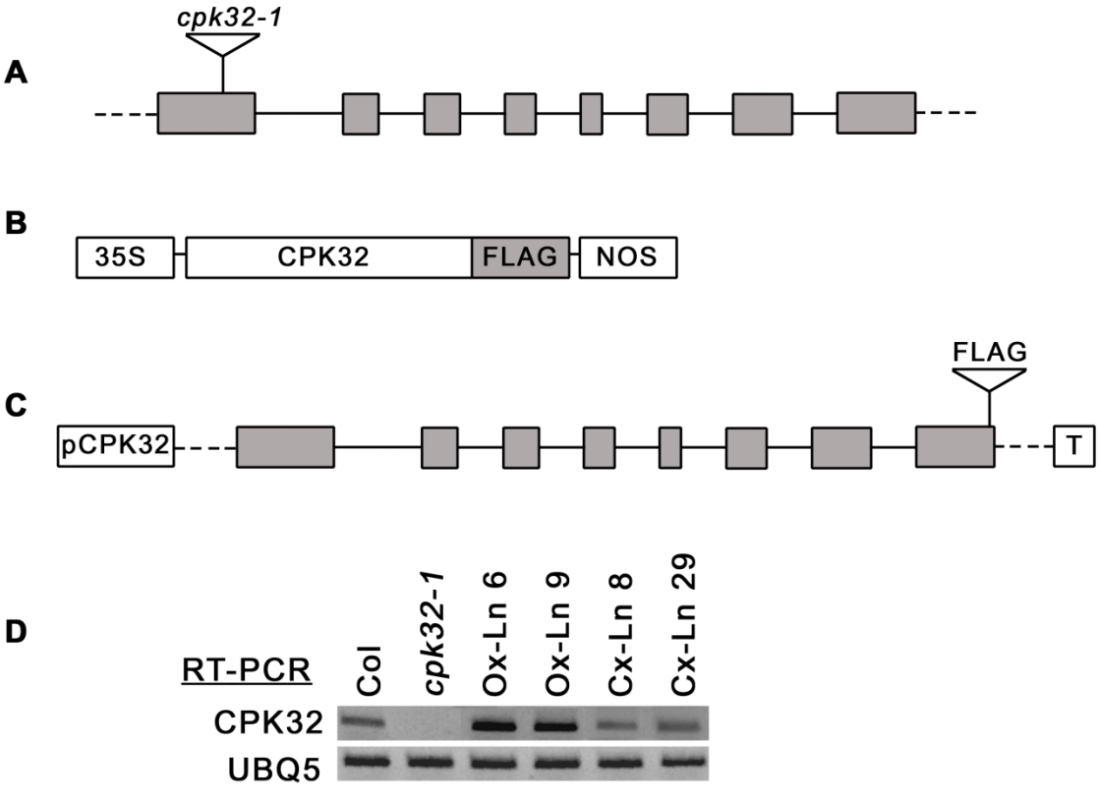

Figure 2.5. Molecular characterization of CPK32 mutant and transgenic lines. A, Gene structure of CPK32 (At3g57530) and position of the T-DNA insertion in the first exon (open triangle). All boxes and lines are not drawn to scale. Closed boxes represent exons and solid lines represent introns. Dotted lines represent UTRs. B, Plasmid construct used to transform Arabidopsis (Col-0) to obtain Ox-Ln 6 and Ox-Ln9 lines. 35S represents the CaMV promoter and NOS represents the nopaline synthetase terminator and FLAG represents the 8 amino acid FLAG tag. C, Structure of the plasmid construct used to complement *cpk32-1* mutant lines. The pCPK32 refers to as 2 kb promoter of CPK32. T represents the 500 bp terminator region. The open triangle represents the inserted FLAG tag. D, RT-PCR analysis of CPK32 transcript levels in seedlings of indicated genotypes. Amplified ubiquitin5 (UBQ5) was used as control.

Four homozygous lines were obtained from 12 heterozygous lines. The level of CPK32 transcript was enhanced in all of the overexpression lines as compared to WT plants (e.g., Ox-Ln6 and Ox-Ln9; Fig. 2.5D). Leaves of these two transgenic lines also were used in immunoblot analysis to determine whether they express the CPK32 fusion protein. When used in Western blot analysis with anti-FLAG antibody, both lines expressed CPK protein from the introduced gene (Fig. 2.6C).

The *cpk32-1* mutant plants were transformed with a construct containing the full length CPK32 gene, as shown schematically in Fig. 2.5C. A FLAG epitope tag was introduced upstream of the stop codon to monitor expression of CPK32 protein in the complementation lines. After screening for antibiotic selection, we obtained four homozygous lines from fourteen heterozygous lines that showed 3:1 segregation. Semi-quantitative RT-PCR analysis was performed using RNA isolated from these complementation lines along with control Col plants. Homozygous lines (Cx) had WT levels of CPK32 transcript (Fig. 2.5D), confirming that CPK32 gene expression was restored in these lines. Similarly, CPK32 protein level in the complementation lines was analyzed by Western blot (Fig. 2.6C). Using the anti-FLAG antibody, CPK32 protein levels were detected in the complementation lines as well.

We also evaluated CPK32 protein expression in the different genotypes using an anti-CPK32 polyclonal antibody. This antibody was made against a 20 amino acid peptide from the N-terminal variable region of CPK32, 22RKKQNPFSIDYGLHHGGGD40. This region is highly specific to CPK32. We examined all other Arabidopsis CPKs and found no substantial homology to this

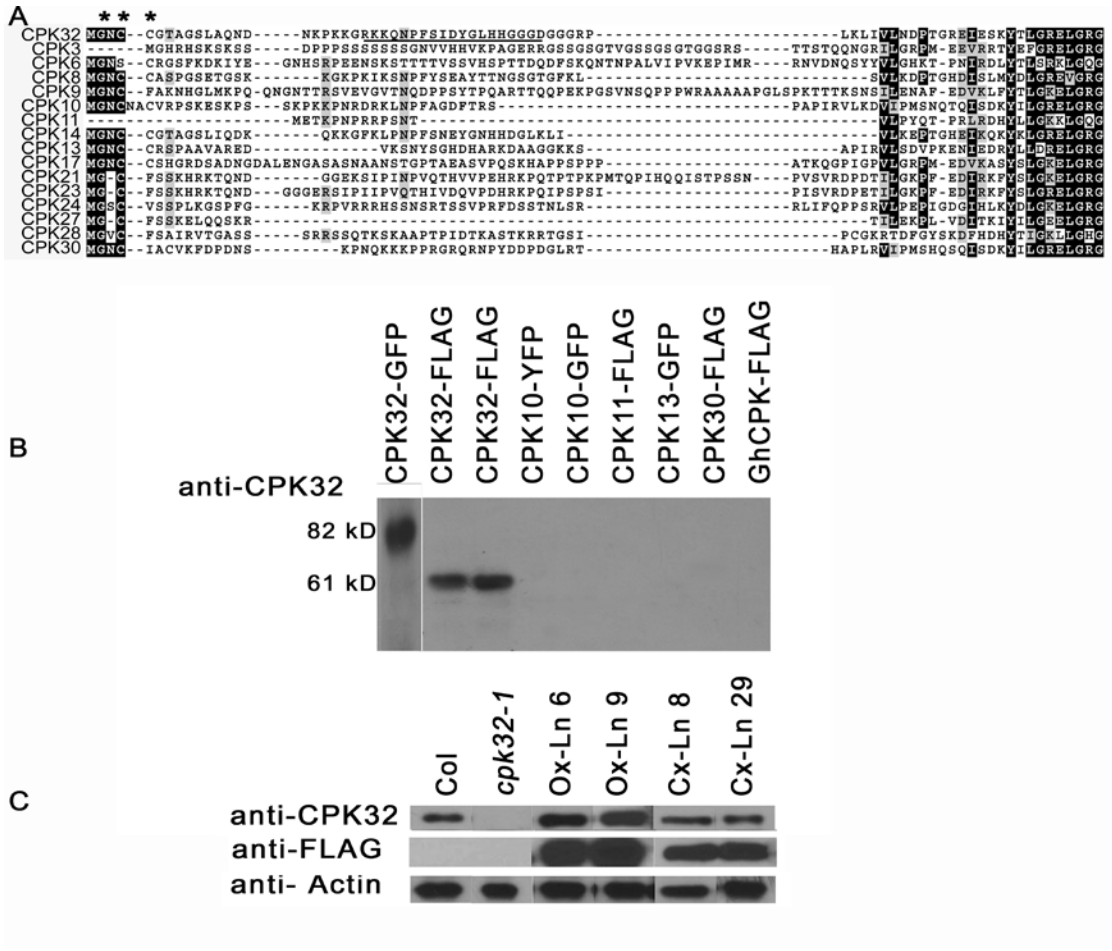

Figure 2.6. Western blot analysis of expressed CPK proteins. A, Alignment of the N-terminal amino acid sequence from sixteen representative CPKs. The underline indicates the amino acid sequence used in making anti-CPK32 polyclonal antibody. The asterisks show the N-terminal Gly and Cys residues, which are predicted to be myristoylated and palmitoylated, respectively. B, Western blot of proteins expressed in maize protoplasts by transient expression and probed with anti-CPK antibody. The molecular weights of these CPKs range from 50 to 85 kD. Membrane was stripped and re-probed with anti-FLAG antibody to confirm the expression of FLAG tagged proteins (data not shown). C, Western blot using anti-CPK32 (upper panel) and anti-FLAG antibodies (lower panel) after gel electrophoresis of 2 μg soluble protein from seedlings of WT, *cpk32-1*, two CPK32 overexpression lines (Ox-Ln6 and Ox-Ln9) and two complementation lines (Cx –Ln8 and Cx-Ln29). Anti-actin was used as loading control (middle panel).

peptide (*e.g.*, Fig. 2.6A). We tested the specificity of this antibody by Western blots in two experiments.

First, we expressed CPK32 and five other CPKs in maize protoplasts and tested their cross-reactivity (Fig. 2.6B). Only the expressed protein from the CPK32 clone was cross-reactive. Next, we tested for possible cross-reactivity with proteins from the *cpk32-1* seedlings (Fig. 2.6C). We observed no product of the correct size in the mutant, but we did observe in WT extracts a cross-reaction with a protein of about 63 kD. We then tested for protein expression in the overexpression and complementation lines. Both Ox-Ln6 and Ox-Ln9 had enhanced levels of CPK32 protein levels relative to WT, while Cx-Ln8 and Cx-Ln29 had cross-reacting protein at a level similar to that which occurred in WT plants.

Stress Response Phenotypes of CPK32 Overexpression and Mutant Lines

Since the CPK32 transcript accumulated following a variety of stress treatments, we investigated whether the mutant and transgenic lines with altered protein expression display any stress related growth phenotypes. Under normal growth conditions, *cpk32-1*, Ln29). Anti-actin antibody was used as loading control (middle panel). Ox, and Cx lines did not show any visible phenotypes in growth pattern, plant size, flowering time, seed production, or viability (data not shown). We first tested the influence of NaCl on germination and seedling growth. All genotypes showed 100% germination on plates without salt. However, there were significant developmental differences between the

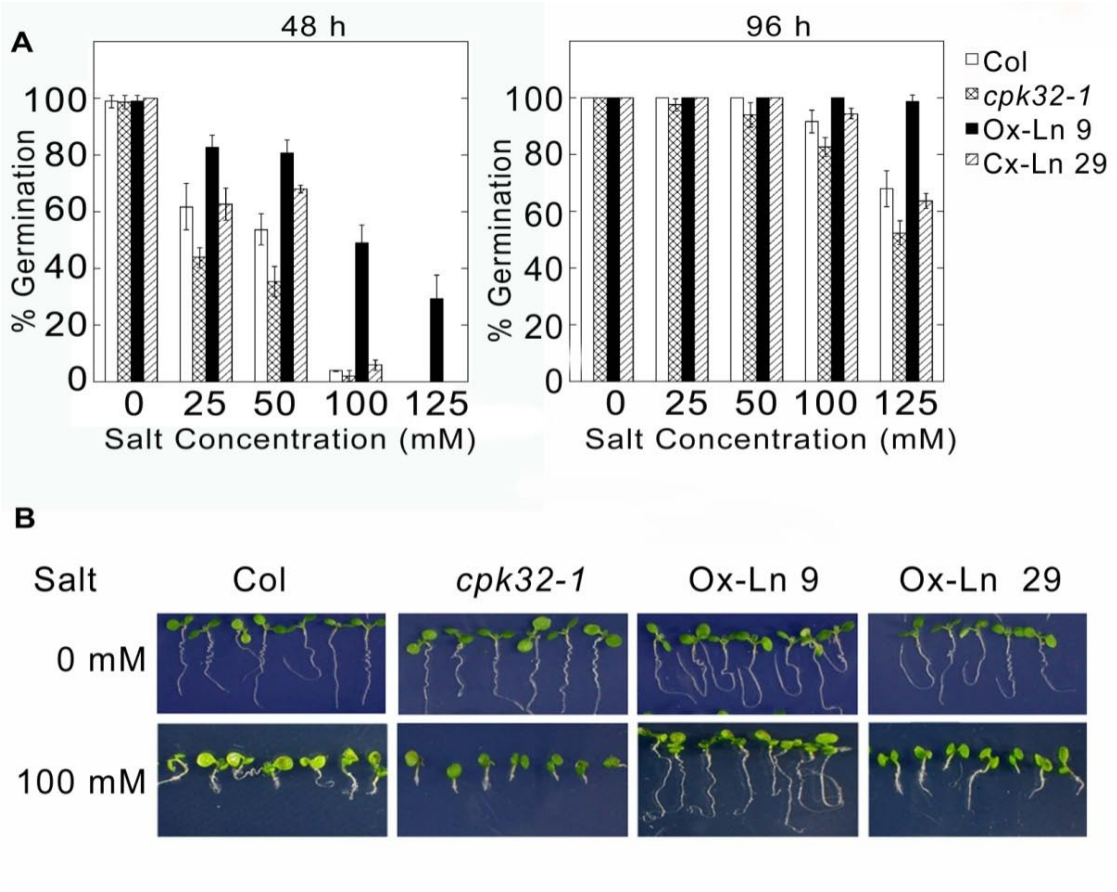

Figure 2.7. CPK32 mutant and transgenic lines show salt dependent phenotypes. A, Salt hypersensitive phenotype of *cpk32-1* and relatively insensitive phenotype of Ox-Ln9 in germination assays. Seeds from each genotype were germinated on MS plates with or without NaCl. Germination was scored after 48 h and 96 h. All values show mean ± SD from three replicates. The entire experiment was repeated three times. B, Seedling growth on 100 mM NaCl. The top panel shows seedlings grown for 7 d without salt. The lower panel shows seedlings grown on 100 mM salt. The Ox-Ln9 seedlings are very tolerant to salt, whereas *cpk32-1* seedlings are hypersensitive to salt treatment. Similar results were obtained using Ox-Ln6, Ox-Ln12, Ox-Ln15 and Cx-8, Cx-Ln14, Cx-Ln 21.

genotypes as soon as 48 h after plating on salt. At this time, Col seeds had 62% germination on plates supplemented with 25 mM NaCl. However, seeds of Ox-Ln9 were relatively insensitive with 82% germination (Fig. 2.7A). Seeds of *cpk32-1* were hypersensitive to salt stress and showed a lower germination rate of 43% (Fig. 2.7A). With increasing salt concentrations from 25-125 mM, the Ox-Ln9 seeds showed a relatively insensitive phenotype (Fig. 2.7A). Interestingly, the hypersensitive phenotype of *cpk32-1* was restored in seeds from the complementation lines. These seeds showed WT sensitivity in the germination assay at all salt concentrations (Fig. 2.7A).

We also tested the influence of 0.1 M NaCl on seedling growth. In this condition the Ox-Ln9 line was relatively salt tolerant, (Fig. 2.7B) with rather normal root growth. However, the post germination growth of *cpk32-1* seedlings was significantly reduced in the presence of this level of salt. The cotyledons and roots of *cpk32-1* mutant seedlings were much smaller than those of Col or of the complementation lines. The latter 2 lines had very similar growth responses.

CPK32 Mutant and Transgenic Plants Show Altered Sensitivity to ABA

Abiotic stresses such as salt can be mediated by both ABA-dependent and ABA-independent response pathways (Mahajan and Tuteja, 2005). We therefore examined whether ABA has an effect on the germination rate or on the post-germination growth of all CPK32 genotypes. Since ABA is known to delay germination, we studied seed germination of all the genotypes in a time dependent manner on plates containing 0-0.8 μM ABA. Germination was scored every 24 h starting 48 h after plating. All seeds

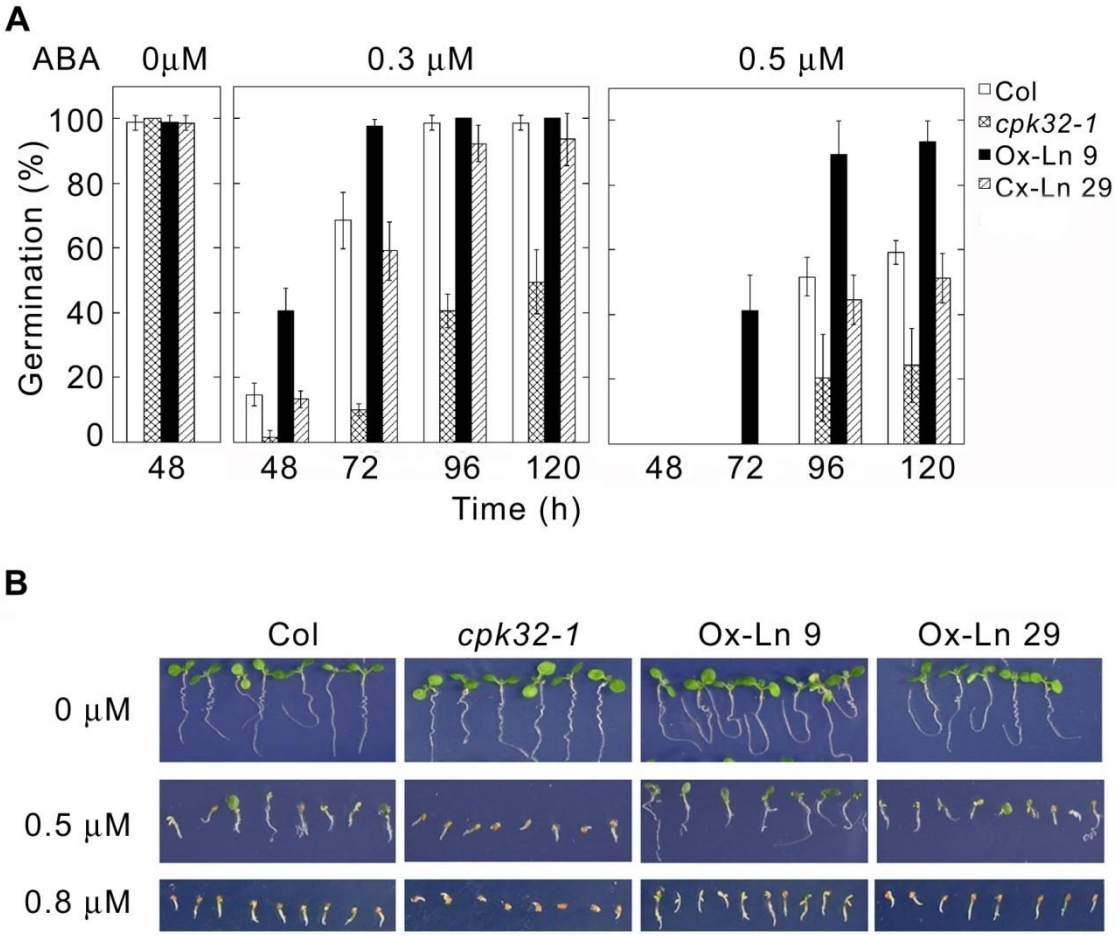

Figure 2.8. ABA sensitive phenotypes of CPK32 mutant and transgenic lines. A, Germination of CPK32 genotypes and WT on plates containing 0 µM ABA (left), 0.3 µM ABA (middle) and 0.5 µM ABA (right). Germination was scored after 48 h intervals. Values shown in graphs are means ± SD from 3 replicates. B, Growth phenotypes of CPK32 mutant and transgenic lines on plates containing 0.5 and 0.8 µM ABA. Pictures were taken after 7 d from planting seeds on plates. The entire experiment was repeated three times. Similar results were obtained using other Ox and Cx lines as mentioned in Fig.2.7.

showed almost 100% germination after 48 h on plates without ABA (Fig. 2.8A). Seeds of both WT and complementation lines had reduced germination rates to only 16% after 48 h on plates containing 0.3 μM ABA. Seeds of Ox-Ln9 were less sensitive to ABA, with a germination rate of about 40% after 48 h. However, germination of Ox-Ln9 was still sensitive to ABA. In contrast, seeds of *cpk32-1* were ABA hypersensitive, with only 1-2% germination on 0.3 μM ABA plates (Fig. 2.8A). On plates with 0.5 μM ABA, seeds of WT, *cpk32-1*, Cx-Ln29 failed to germinate after 48 h, but did so to varying extents thereafter. These observed dose and lineage-dependent responses were maintained throughout the 120 h test period. The Ox-Ln9 seeds more quickly reached full germination in the presence of ABA. Therefore, CPK32 might act as a negative regulator of ABA-dependent repression of seed germination.

When all the genotypes were allowed to continue growing for 7 d on ABA containing media, Col seedlings grew at a slower rate and were smaller than Ox-Ln9 seedlings. Growth of the *cpk32-1* seedlings was severely impacted by ABA. These plants were not able to form cotyledons, hypocotyls or roots on plates supplemented with 0.5 μM or 0.8 μM ABA (Fig. 2.8B). On the other hand, Ox-Ln9 seedlings could differentiate these organs when grown on plates with 0.5 μM or 0.8 μM ABA, and formed green cotyledons. Seedlings of Cx-Ln29 had similar growth phenotypes as those of Col. Both WT and Cx-Ln29 seedlings showed organ differentiation on 0.5 μM ABA, but not on 0.8 μM ABA by the end of the 7 d period. These phenotypes indicate that CPK32 has a role in ABA-dependent regulation of post-germination growth. Again, the

data indicate that CPK32 might act as a negative effector of ABA regulation of these early plant growth processes.

Dehydration Stress Sensitivity of CPK32 Mutant and Transgenic Plants

ABA is key regulator of plant growth and development, particularly through its role as a stress hormone that enables plants to respond limiting water. We therefore investigated the sensitivity of our different CPK32 expression lines to imposed dehydration stress. In this experiment, plants were not watered for two wks to simulate drought conditions, as described by Zhu et al. (2007). After 9 d of treatment, about 20% of WT plants showed wilting, as did about 10% of the *cpk32-1* plants (Fig. 2.9A). Interestingly, about 80% of the Ox-Ln9 plants were wilted by 9 d. The complementation line Cx-Ln 29 responded to treatment to a similar extent as did Col. After two wks, plants were re-watered and were scored for continued growth and completion of life cycles. WT and Cx-Ln29 had 47% and 45% survival, respectively; *cpk32-1* plants had 83% survival; while Ox-Ln9 had only 7% survival (Fig. 2.9A). Thus, unlike results from the salt and ABA stress assays, the overexpression of CPK32 made plants hypersensitive to drought stress, whereas the *cpk32-1* plants were relatively insensitive to drought stress.

To investigate whether this hypersensitivity of Ox-Ln9 plants might be associated with an increased number of stomata, we counted the number of stomata per unit area in all genotypes. We used lower epidermal peels of all CPK32 genotypes and observed them under light microscopes. For each genotype we used 5-7 epidermal peels.

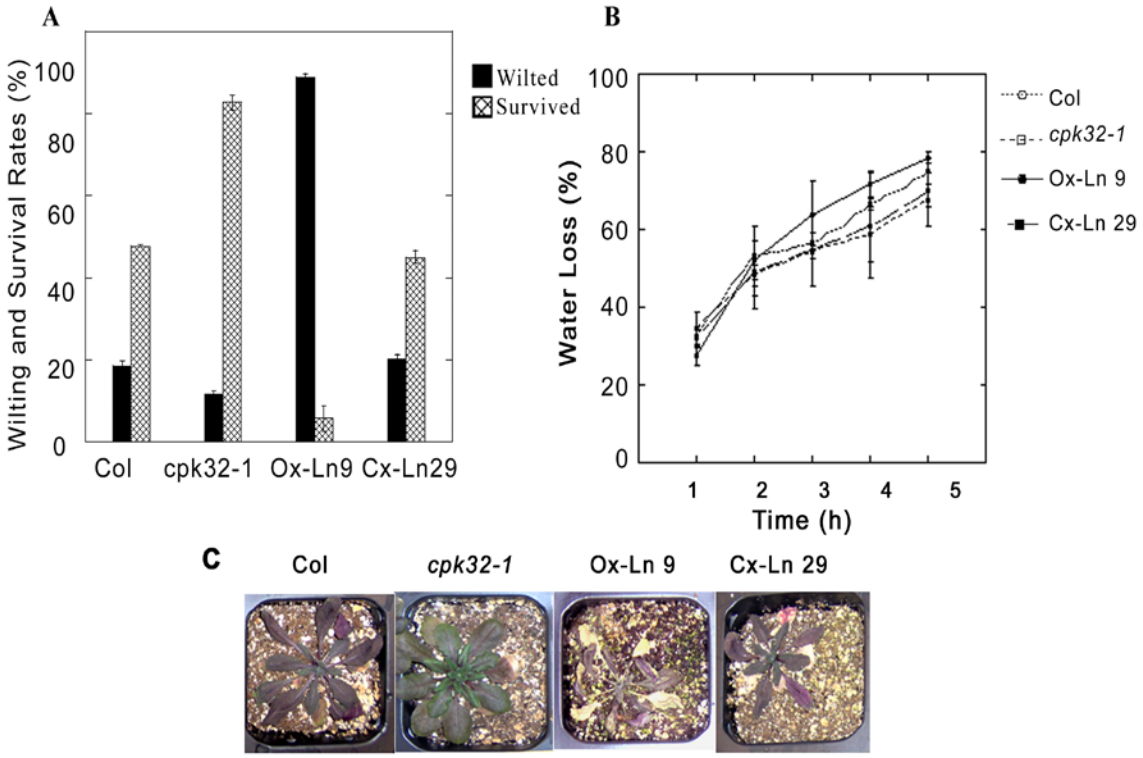

Figure 2.9. Influence of CPK32 expression on plant responses to drought stress. A, To induce drought stress, 3-wk-old chamber grown plants were not watered for two wks. After withholding water for 9 d, plants were scored for apparent wilting (solid bars). After 2 wks, normal watering resumed. Plants that completed their life cycle were scored for survival (hatched bars). B, Rate of water loss from leaves over 5 h periods. Water loss was determined by the reduction in fresh weight of twenty five leaves from each genotype. Values are means ± 1 SD. C, Representative plants of each genotype after two wks of water withholding. Pictures were taken on the last day of drought stress.

However, we did not find any significant change in the number of stomata among the different lines (data not shown). The water loss from leaves was measured as change in fresh weights over a 5 h period as shown in Fig 2.9B. Leaves from Ox-Ln 9 lost water at a faster rate than that of *cpk32-1* mutant. Thus, we surmise that the more rapid rate of water loss from the Ox-Ln9 plants is due to altered stomatal activity. We also tested whether Ox-Ln9 and *cpk32-1* mutant plants are sensitive osmotic stress. For this, we placed seeds of all genotypes on MS plates infused with polyethylene glycol (PEG) solution to induce osmotic stress (Weele et al., 2000). However, we observed ambiguous results in the responses of these genotypes (data not shown).

Expression of Stress Response Genes in CPK32 Mutant and Overexpression Lines

Salt, ABA and drought response phenotypes of the CPK32 mutant and overexpression plants suggest that CPK32 has a role in abiotic stress signaling. To find out whether these stress responses are regulated through changes in ABA response genes, we investigated whether the expression of stress regulated genes is different in *cpk32-1* and Ox-Ln9 seedlings. As stress inducible genes, we selected RD29A, RD22, RAB18, DREB2A, MYC2, and COR 47. These genes have previously shown to be involved in ABA signaling, as well as salt and drought stress responses (Zhu et al., 2007). RD29A is perhaps the most studied stress response gene, and is induced by ABA, salt, and drought (Yamaguchi-Shinozaki and Shinozaki, 2007). We also examined expression of an ABA biosynthetic gene, NCED3, since the CPK32 genotypes could have altered ABA levels.

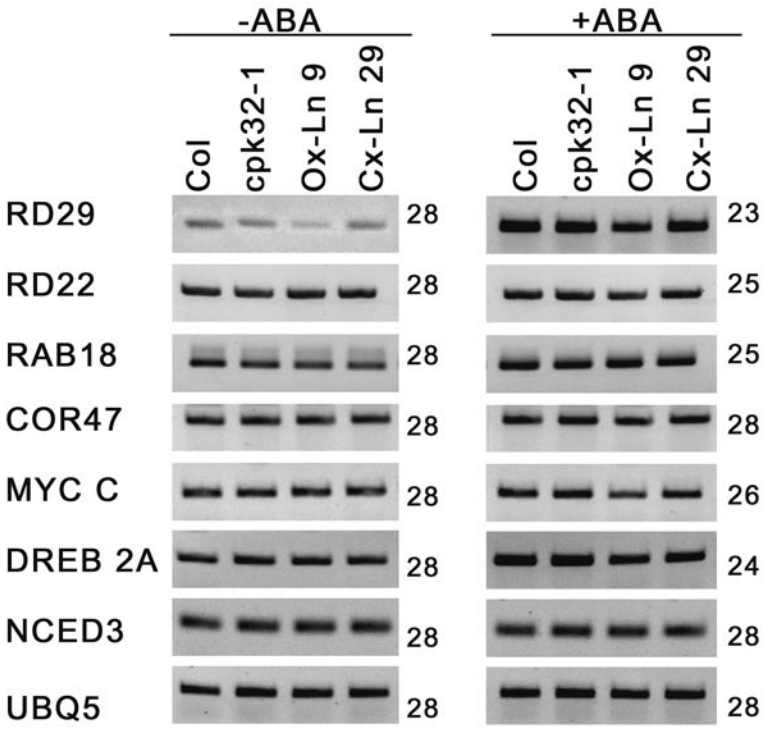

Figure 2.10. Expression of different ABA response genes in CPK32 mutant and transgenic lines. Seedling of Col, , *cpk32-1*, Ox-Ln9, and Cx-Ln29 were grown on agar plates and then treated with water or water with 50 μM ABA for 4 h. Semi-quantitative RT-PCR was used to determine transcript levels of RD29A, RD22, RAB18, COR47, MYC 2, DREB2A, and NCED3. UBQ5 was used as an amplification control. The figure presents the amplification product of only one PCR cycle per gene though the number of PCR cycles was varied for each gene to get optimum product amplification gene.

The expression of stress response genes RD22, RAB 18, DREB2A, and COR 47 was not significantly different among the different genotypes in the absence of ABA treatment (Fig. 2.10). When the plants were treated with 50 μM ABA for 4 h, there was an increase in the expression of all the ABA response genes, but this increase occurred similarly among all of the CPK32 genotypes. In these analyses, we varied the PCR cycle numbers to avoid product saturation and interference from detection limits. Even with lower PCR cycle numbers, there was at best only subtle differences in the expression of these ABA response genes among the different CPK32 genotypes. For example, RD29A was modestly reduced in Ox-Ln9 both with and without ABA treatment. Also, MYC2 showed somewhat less ABA-induced increase in Ox-Ln9. The expression of NCED3 also was unchanged among the CPK32 genotypes, and was not changed after ABA treatment. Thus, these results indicate that CPK32 does not regulate the expression of ABA response genes, except for a modest repression of the RD29A gene and the MYC2. Hence, there must be an alternate mechanism to account for the ABA-dependent phenotypes of plants with altered expression of CPK32.

Sub-cellular Localization of CPK32 Changes in Response to ABA Treatment

Localization studies involving different CPKs indicate that collectively they occur in diverse places including the plasma membrane, cytosol, ER, nucleus, and even oil bodies (Cheng et al., 2002; Harper and Harmon, 2005). This wide distribution could be important for decoding Ca signatures at various cellular sites. To establish where CPK32

is normally expressed, we cloned the gene as a C-terminal fusion with GFP and expressed the plasmid DNA in a protoplast transient assay. The cDNA was transfected into pea leaf protoplasts and allowed to express for 6-8 h, before observing the GFP fluorescence with a confocal microscope. In a sectional view of the protoplast, the CPK32-GFP fluorescence was associated with the cellular periphery, forming a smooth, narrow rim (Fig. 2.11A). This is consistent with a plasma membrane localization. Imaging through a z-stack resulted in many concentric narrow rings of fluorescence, again indicating the plasma membrane localization (data not shown). We also observed the same pattern of GFP fluorescence after plasmid expression in transfected protoplasts from leaves of Arabidopsis, pea or maize (data not shown).

Since several phenotypes of the CPK32 transgenic lines indicated that the protein might have a role in abiotic stress signaling, we examined whether the localization of CPK32 changes in response different treatment. We treated protoplasts expressing CPK32-GFP with varying concentrations of salt, $CaCl_2$, hydrogen peroxide, auxin, ABA, and giberrelic acid. Among these treatments, only ABA affected CPK32 localization. Protoplast treatment with 5 μM ABA rapidly altered CPK32 localization, with an initial extensive internalization of fluorescence into the cytosol within 5-10 min, followed by sequestration of fluorescence into the nucleus after 15-20 min (Fig. 2.11). This internalization of CPK32 and its movement to the nucleus was highly specific to ABA, as other hormones or stress agents were unable to produce a similar response. Treatment with $CaCl_2$, or gibberelic acid, plus ABA failed to produce a different or more rapid localization response than that of ABA treatment alone (data not shown). To confirm the

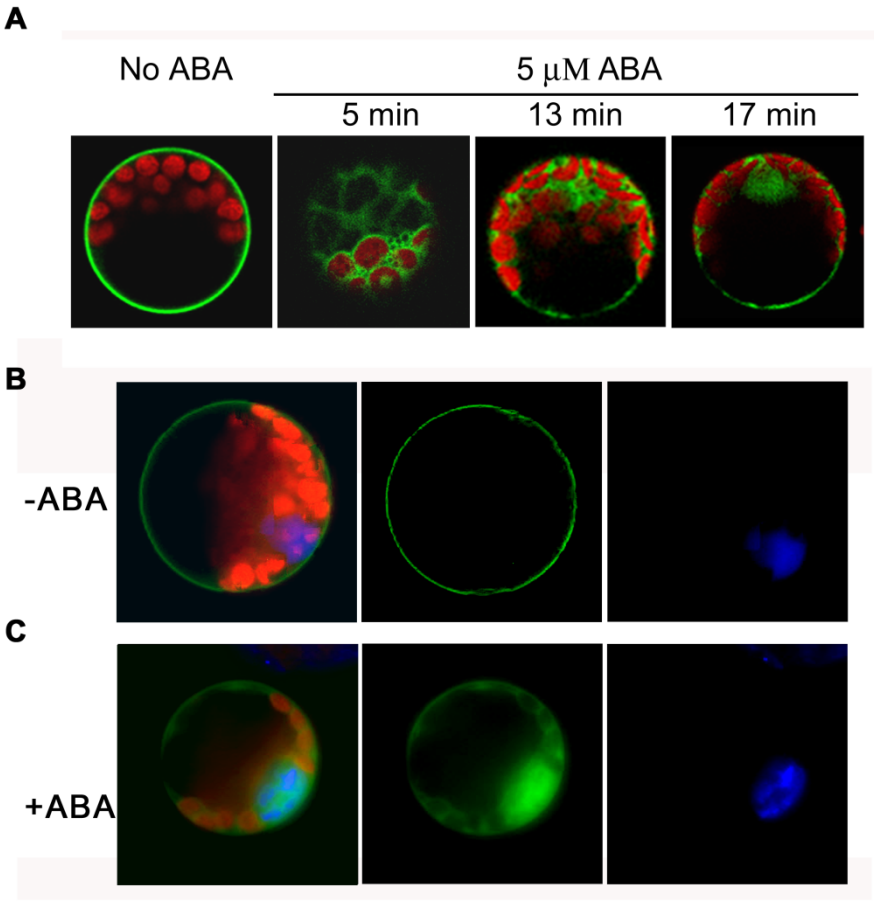

Figure 2.11. The influence of ABA treatment on localization of CPK32 in transfected pea leaf protoplasts. A, In the absence of ABA, CPK32-GFP is expressed at the plasma membrane as seen in far left panel (GFP fluorescence pseudocolored green). The time course shows the altered location of CPK32-GFP after treatment with 5 µM ABA. Chloroplsts show red autofluroscence under GFP filter. Images were taken with a confocal microscope. B and C, In a second experiment, protoplasts were stained with Hoechst dye to show the position of the nucleus (pseudocolored blue) before (B) and after treatment with 5 µM ABA (C). Images were acquired with an epifluorescent microscope.

cytoplasmic localization, we compared the fluorescence with protoplasts transfected with yeast hexokinase2, which targets to the cytosol (Balasubramanian et al., 2007) and shows a similar fluorescence pattern.

CPK32 Localization Requires Post-Translational Lipid Modifications

Most CPKs, including CPK32, are localized to the plasma membrane. This membrane localization of CPKs is attributed to two post-translational lipid modifications, myristoylation and palmitoylation (Cheng et al., 2002). These types of lipid modifications have been shown to be important for a membrane association of numerous proteins. The reversibility of palmiltoylation is thought to contribute to some proteins being able to relocate within the cell (Towler et al., 1988 and Nadolski and Linder, 2007). The N-terminal variable domain of CPK32 includes a potential myristoylation site (Gly-2) and two palmitoylation sties (Cys-4, Cys-5 Fig. 2.12A). We first used direct isotope labeling using [^{3}H]myristic acid and/or [^{3}H]palmitic acid to show that WT CPK32 expressed in protoplasts can be modified by both myristoylation and palmitoylation (Fig. 2.12D). We then used site directed mutagenesis to change the putative myristoylation site (G2A) and the putative palmitoylation sites (C4A/C5A) in CPK32-FLAG, either separately for the 2 modifications or with all changed in one construct (G2A/C4A/C5A). Expression of the recombinant proteins was first established using [^{35}S]Met labeling of the newly synthesized modified as palmitoylation site(s) in CPK32 (Fig. 2.12D).

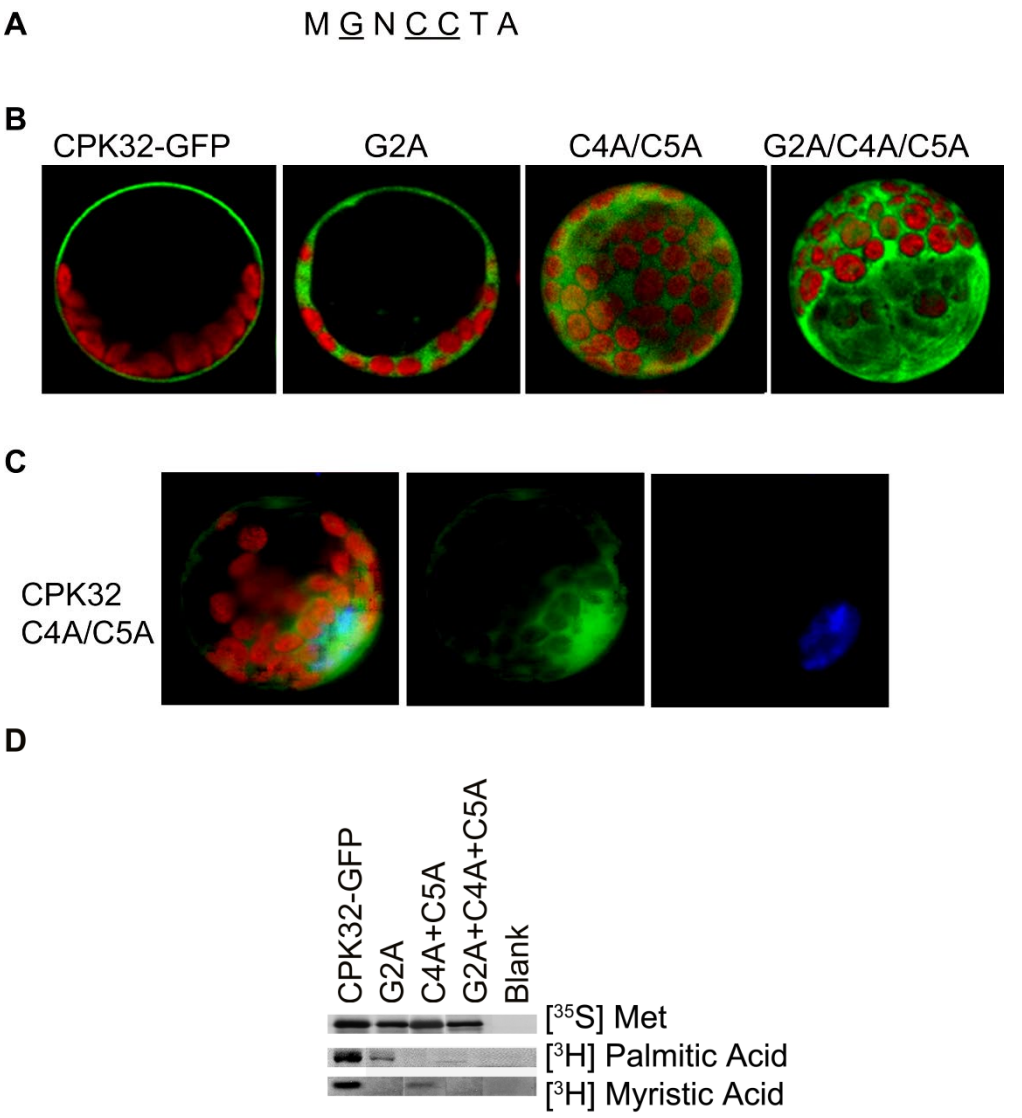

Figure 2.12. CPK32 requires myristoylation and palmitoylation. A, The N-terminal protein sequence showing Gly (G) at 2nd position and 2 Cys (CC) at positions 4 and 5 as myristoylation and palmitoylation sites respectively. B, Protoplasts expressing wild type and mutant constructs. CPK32 wild type shows plasma membrane localization, G2A mutant shows partial cytosolic localization, and C4A/C5A and the double mutant $Cys^{4/5}$ are singly, or both, modified as palmitoylated site(s) in CPK32 (Fig. 2.12D) show cytosolic localization of CPK32 as seen by GFP fluorescence. C, CPK32 palmitoylation mutant (C4A/C5A) showing cytosolic and nuclear localization. Nuclear localization is confirmed by staining

nuclei using Hoechst dye. D, Immunoblots showing [^{35}S] labeled wild type and mutant proteins as an expression control (top panel). Myristoylation and palmitoylation of CPK32 protein is revealed by [^{3}H] labeled myristic acid and [^{3}H] palmitic acid. All proteins were immunoprecipitated using anti-FLAG antibody.

We next investigated the possible role of myristoylation and palmitoylation in the sub-cellular expression of CPK32-GFP, after sub-cloning the site-directed modified CPK32 constructs. When expressed in protoplasts, the G2A construct showed decreased association with the plasma membrane (Fig. 2.12B). The G2A construct showed cytosolic fluorescence in the cytosol, but with a substantial retention of fluorescence at the plasma membrane. The C4A/C5A construct showed a more pronounced cytosolic localization. The myristoylation and palmitoylation triple mutant (G2A/C4A/C5A) had a complete loss of membrane localization. In addition to the cytosolic localization of the triple mutant, fluorescence was also observed in the nucleus. Palmitoylation being reversible also contributes towards movement of proteins between organelles (Towler et al., 1988 and Nadolski and Linder, 2007). The N-terminal variable domain of CPK32 includes a nuclear localization signal (Choi et al., 2005). Thus, we conclude that CPK32 requires myristoylation and palmitoylation for proper membrane targeting and changes in these lipid modifications results in the cytoplasmic and nuclear distribution of CPK32-GFP protein.

CPK32 Acts as a Negative Regulator of RD29 Expression

In order to gain further insight on how CPK32 might function in ABA signaling, we used a protoplast transient expression assay to test whether CPK32 affects the expression of the RD29A gene, a key effector in ABA signaling. Arabidopsis protoplasts were co-transfected with two promoter constructs, pRD29A-LUC construct and as an

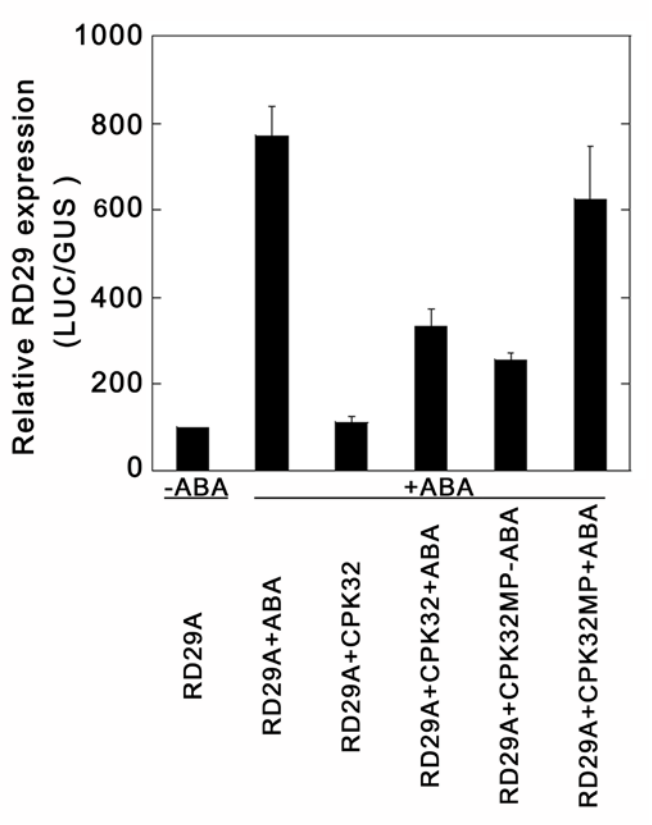

Figure 2.13. CPK32 negatively regulates ABA dependent expression of RD29A. Protoplasts from Arabidopsis were co-transfected with pRD29A-LUC and pUBQ10-GUS constructs. WT CPK32, CPK32 myristoylation and palmitoylation mutant (M+P; G2A/C4A/C5A), and ABF4 were used as effectors. Following transfection protoplasts were treated with 0 or 10 μM ABA. Values are means ± SD for LUC units normalized to GUS activity. GUS activity was not affected by ABA treatment. For the control condition, protoplasts expressed RD29A without ABA treatment and without effectors. Experiments were done in triplicates.

internal control, pUBQ10-LUC. Different possible effector proteins included in the transfections were: WT CPK32-FLAG, CPK32-FLAG triple mutant (G2A/C4A/C5A), and/or ABF4-GFP. RD29A luciferase activity was measured after co-treatment of protoplasts with 0 or 10 μM ABA, in the absence or presence of the expressed effector proteins. We observed that ABA induced RD29A by more than 7-fold (Fig. 2.13). However, WT CPK32 substantially repressed the ABA-dependent induction of RD29A. This lack of induction of the RD29A promoter activity by CPK32 was not proper initial protein targeting to the plasma membrane. Similar results were also obtained while using pea mesophyll protoplasts (data not shown). We then tested whether the transcription factor ABF4 might affect ABA induction of RD29A promoter activity. Expression of ABF4 had no effect on ABA-induction of RD29A, unless the CPK32 mutated protein was also present (Fig. 2.13). In this case, the mutated CPK32 acted synergistically with ABF4 to further increase the ABA-dependent expression of RD29A. These data suggest that CPK32 acts as a negative regulator of ABA signaling mediated by RD29A, but that the de-myristoylated and de-palmitoylated CPK32 protein can act as a positive regulator for RD29A expression.

CPK32 Interacting Proteins

CPKs are good candidates for interacting with a wide range of proteins since they occur in many cellular locations (Cheng et al., 2002; Hrabak et al., 2003; Asano et al., 2005 ; Ray et al., 2007; Li et al., 2008). In order to identify proteins that might be

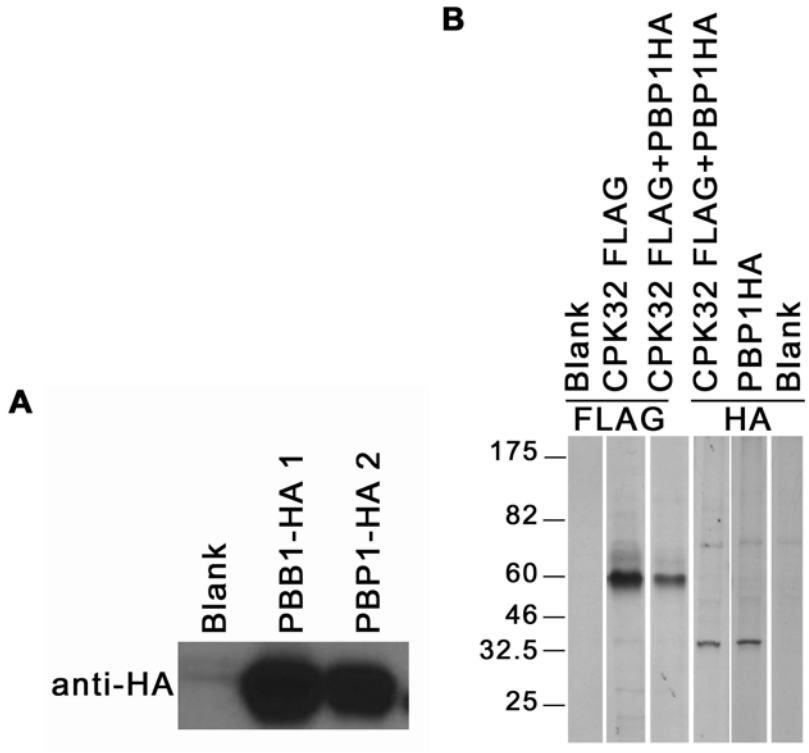

Figure 2.14 Co-immunoprecipitation assay to test for the possible interaction between CPK32 and PBP1 after expression in protoplast transient assay. A, PBP1 was expressed in protoplast transient assay and the expression was confirmed with immunoblotting using anit-HA antibody. B, Immunoblots showing [^{35}S] labeled PBP1 and CPK32-FLAG. PBP1 and CPK32-FLAG were either used in single separate transfection or both co-transfected together. Proteins were co-immunoprecipitated using anti-FLAG or anti-HA antibodies.

interacting with CPK32, we first we Blue-Native (BN) gel electrophosresis initially with extracts of the CPK32-FLAG transgenic line (Ox-Ln9). First, we optimized detergent extraction for maximum recovery of CPK32 protein. By immunoblot analyses of the native gels, we found that dodecyl maltoside was more effective than were Triton X-100 or CHAPS (Appendix B). We observed a 550 kD complex that was superimposed with Rubisco, using Blue Natve electrophoresis (Appendix B, Fig. A) hence we used co-immunoprecipitation as an alternative approach. The After detergent titration experiments, we used 900 µg of soluble protein, 2 µl of anti-FLAG antibody, followed by capturing possible immunocomplexes using protein A agarose beads. We also used seedling treated without or with ABA to find out whether ABA can lead to differential bands in CPK32 overexpression lines. However, in these experiments gels stained with Colloidal Coomassie blue did not show any unique protein bands not otherwise observed in extracts processed from WT or *cpk32-1* seedlings (Appendix B, Fig. B,C).

As an alternative approach, we substituted anti-CPK32 antibody for immunocapture. In this case, we found two differentially expressed protein bands, one at ~32 kD and another at ~80 kD (Appendix B, Fig. C). The 32 kD protein was overexpressed in *cpk32-1* seedlings, while the 80 kD band was overexpressed in the Ox-Ln9 seedlings (Fig. 2.14B). Both protein bands were excised from gels and submitted for micro-sequencing. The 32 kD protein was identified as a PYK10 binding protein (PBP1, At3g16420), while the 80 kD protein was not identified. PBP1 belongs to the jaclin-lectin family of proteins. It binds to inactive form of PYK10 and acts as a molecular chaperon to polymerize active and inactive forms of PYK10 upon pathogen attack. Since

CPK32 is regulated by biotic stress stimuli, PBP1 was considered to be a feasible interacting protein.

First, we checked the transcript abundance of PBP1 in CPK32 mutant and overexpression lines. We did not observe any change in the expression of CPK32 transcript in absence or in presence of ABA in CPK32 mutant and transgenic lines (Appendix C). Therefore we suggest that CPK32 does not transcriptionally regulate expression of PBP1. In order to directly test a putative interaction between CPK32 and PBP1, we cloned PBP1 as a fusion protein with a double HA tag. The PBP1-HA protein was expressed in protoplasts, as shown by a Western blot using anti-HA antibody (Fig. 2.14A). A co-immunoprecipitation assay was done by co-expressing CPK32-FLAG and PBP1-HA in maize protoplasts. However, the PBP1 and CPK32 proteins were not able to immunoprecipitate the partner protein using corresponding antibodies (Fig. 2.14B). Thus, these results indicate that CPK32 and PBP1 probably do not physically interact with each other. Therefore, the reason that PBP1 accumulates in *cpk32-1* is not known.

DISCUSSION

Arabidopsis genome encodes thirty four CPKs. Most of these CPKs remain to be functionally characterized. Due to expected functional redundancy and lack of single CPK mutant phenotype, it has been challenging to assign function to each CPK. Here we report functional characterization of CPK32 from Arabidopsis. In this study we have functionally characterized CPK32 using genetic and cellular approaches. To understand the function of CPK32, we first analyzed the tissue specific expression of CPK32 using RT-PCR analysis. The results of RT-PCR from various plant tissues indicated that CPK32 is expressed in all plant parts with high levels of expression in seedlings, roots, inflorescence, and germinating seeds. Tissue specific expression using pCPK32-GUS plants showed results similar to RT-PCR analysis. The CPK32 promoter is expressed in roots, more specifically in root cortex and root cap, pollen, embryo, leaf hydathodes, phloem tissue, and abscission zone of siliques. Our results support the findings of a microarray analysis of Arabidopsis which indicated that CPK32 is expressed in almost all plant organs (PlantsP; http://plantsp.genomics.purdue.edu). Previous study on the CPK32 promoter expression by Choi et al. (2005) also showed that CPK32 is expressed in these organs. Our results suggest that CPK32 is not expressed in leaves except for hydathodes unless the leaves are stressed. When pCPK32-GUS plants were challenged with abiotic or biotic stresses, we did observe an increase in CPK32 promoter activity in leaves (Appendix A). Our results indicate that CPK32 is expressed in those organs which are either involved in osmo-sensing such as roots, or those which undergo dehydration

and/or rehydration processes like pollen. The root cap is primarily involved in sensing gravity through accumulation of starch in the amyloplast, osmo-sensing, and sensing high salinity in the rhizosphere (Eapen et al., 2003; Li and Zhang, 2008). The roots also accumulate more ABA in response to salt stress through anosmo-semsing mechanism (Jia et al., 2002). Thus, the expression of CPK32 in root cap might have a role in sensing water availability. Alternatively, CPK32 might be important for osmo-sensing and/or sensing salinity. Similarly, the expression of CPK32 in hydathodes is another example of CPK32 being associated with tissue that is involved in regulating excretion of water, salts, sugars, and organic substances. Mature pollen undergoes extensive dehydration processes during maturation and rehydrates in order to germinate (Barnabas and Rajki, 1981). A similar process of dehydration and rehydration is seen in seeds during maturation, dormancy, and germination. Pollen and seeds both are also associated with the storage of many metabolites and nutrients. Expression of CPK32 in the phloem of conducting tissues supports the idea of the CPK32 is associated with nutrient management. Thus, the expression pattern of pCPK32 indicates that CPK32 is somehow associated with organs that are involved in regulating water and nutrients in plants. Based on our results of CPK32 expression, we speculate that CPK32 might have a role in physiological aspects of dehydration, osmotic regulations, and nutrient management in Arabidopsis.

To refine our knowledge about CPK32 expression, we used publicly available databases and identified *cis* acting elements in the CPK32 promoter. Interestingly, the analysis of the CPK32 promoter using the PLACE database, revealed the presence of

many elements that are regulated by abiotic and biotic stresses. The CPK32 promoter contains regulatory elements such as ABRE, ABRE-CE, and binding sites for several transcription factors that are involved in stress responses through ABA signaling (Table 2.4 and 2.5). Also, there are six W-Box motifs in pCPK32 required for binding of WRKY transcription factors that are known to regulate bacterial and fungal pathogen responses (Asai et al., 2002), senescence (Robatzeket al., 2001), wounding, pathogen defense response and drought responses (lker and Somssich, 2004; Lippock et al., 2007). The data from pCPK32-GUS (Fig. 2.3) as well as transcript abundance using qPCR (Fig. 2.4), shows that CPK32 is upregulated by abiotic as well as biotic stress stimuli. Our data shows that CPK32 is regulated by many stress stimuli, which might be due to the abundance of these regulatory elements.

ABA plays an important role in regulating seed dormancy, seed germination and seedling growth as well as responses to abiotic stresses such as drought or salt stress (Schroeder et al., 2001; Finkelstein and Rock, 2002). In addition to the up-regulation of CPK32 by ABA, salt, and osmotic stress, our results from phenotype assays indicate that the gain of function transgenic lines and the loss of function mutants have altered sensitivity to ABA, salt, and drought stress. The overexpression lines are relatively insensitive to ABA whereas the *cpk32-1* mutants are hypersensitive to ABA in germination and in early seedling growth assays. These hypersensitive phenotypes of *cpk32-1* were complemented with the native CPK32 gene confirming, the role of CPK32 in ABA responses.

ABA also regulates physiological responses to many abiotic and biotic stresses including salt stress, cold, drought, osmotic stress, wounding, and pathogen responses (Leung and Giraudat, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). Since Ca^{2+} acts as a second messenger in several signaling pathways, it is possible that CPKs may be involved in multiple stress signaling pathways. In our study, we found that along with ABA insensitivity, CPK32 overexpression lines were also insensitive to salt, whereas *cpk32-1* was hypersensitive. Nuclear protein X1 (NPX1) has recently been shown to act as a negative regulator of ABA signaling. It is upregulated by ABA, salt, and dehydration and its overexpression results in ABA and salt insensitive phenotypes. But, the overexpression of NPX1 leads to a hypersensitive response to drought stress due to altered ABA-induced stomatal movement (Kim et al., 2009). CPK32 transgenics have similar phenotypes as those of NPX1 in ABA, salt, and drought stress responses. In contrast to ABA and salt stress assay, CPK overexpression lines were hypersensitive to drought stress and *cpk32-1* mutants were insensitive to drought. During drought stress the ABA concentration in leaves increases and leads to the closing of stomata, thus minimizing water loss (Nilson and Assman, 2007). Since there was no difference in the number of stomata among overexpression, *cpk32-1* mutant, and wild type plants, different rates of water loss from the leaves can be attributed to a possible alteration of stomatal activity. Similar phenotypic responses have been observed for these, including Receptor for activated C kinase 1 (RACK1; Guo et al., 2009), CBL1 and its interacting protein kinase (PKS3; Guo et al., 2002), and G- protein coupled receptor GCR1. The drought insensitive phenotype of RACK1, CBL1, and GCR1 transgenic over expression

lines that also show ABA and/or salt hypersensitivity have previously been linked stomatal activity (Pandey and Assman, 2006). Since CPK32 overexpression plants are relatively insensitive to exogenously applied ABA in germination and growth assay, it is possible that these plants are also insensitive to increased ABA levels in guard cells during drought stress, leading to decreased water loss from the leaves of overexpression plants. In contrast, *cpk32-1* plants which are hypersensitive to ABA might respond to even slightest increase in ABA concentration due to drought and close stomata leading to conservation of water loss, giving a drought resistant phenotype. Thus, together the ABA and salt insensitive phenotypes of CPK32 overexpression plants indicate that CPK32 might act as a negative regulator of ABA dependent stress signaling. CPK32 is not transcriptionally upregulated by dehydration and *cpk32-1* mutant shows a drought resistant phenotype. Hence, CPK32 may have a separate role in drought signaling that is seperates from ABA related stress signaling at some point in the signal transduction process.

Previously, Choi et al. (2005) have shown that in a Yeast two hybrid assay, CPK32 interacts with the bZIP type ABRE binding factors, ABFs such as ABF1 to 4. We co-expressed CPK32-FLAG and ABF4-GFP in protoplast transient expression assay and co-immunoprecipitated both CPK32 and ABF4 using anti FLAG and anti-GFP antibodies respectively. In our pull down assays we did not find interaction between CPK32 and ABF4. They also claim that other ABFs from the same group, ABF1, ABF2, and ABF3. But, using co-immunoprecipitation approach we did not find interaction between CPK32 and ABF4. Also, Choi et al. (2005) claimed that the overexpression

lines of CPK32 shows insensitivity to salt and ABA. In ABA germination assay, they did not observe the insensitive phenotype on 0.5 µM and 1 µM ABA. Even on the very high concentration of ABA, such as 2 and 5 µM ABA, the data provided does not show significant difference between wild type (Ler) and the overexpression lines (Fig 6 C and D Choi et al., 2005). The lack of experimental details leaves uncertainty about the insensitive phenotypes of CPK32 overexpression lines. The results for germination assay in presence of salt also have similar experimental shortcomings, though the overexpression lines show a trend that suggests insensitive phenotypes. But, our data suggest that CPK32 has a role in ABA signaling and also affects salt as well as drought responses. Overexpression of CPK32 leads to ABA and salt insensitive phenotypes whereas the *cpk32-1* shows hypersentive phenotypes in germination and early growth assays. Mutation in CPK32 leads to drought sensitive phenotypes. Salt, ABA, and drought sensitive phenotypes of *cpk32-1* mutant are complemented with native CPK32 gene.

The altered phenotypic responses to ABA, salt and drought can result from regulation of multiple stress response genes, such as transcription factors, early stress response genes and/or downstream targets of these products (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2007). Our results from the gene expression studies showed that gain or loss of function of CPK32 does not affect transcriptional regulation drastically as seen by other negative regulation of stress signaling. But the possibility of regulation of these genes by several intermediates that are regulated by CPK32 cannot be ruled out. On the other hand, CPK32 might be modulating ABA signaling at the post-

translational level by phosphorylation and de-phosphorylation or through interaction with other stress regulated proteins.

A number of proteins such as McCPK1 and phytochromes A and B are known to move to the nucleus in response to external stimuli (Patharkar and Cushman, 2000; Kircher et al., 1999). McCPK1 has been shown to dissociate from plasma membrane and move into the nucleus via the cytoskeleton in response to salt stress and is thought to phosphorylate a transcription factor that somehow regulates stress response genes (Patharkar and Cushman, 2000; Chehab et al., 2004). To gain further insight into the function of CPK32 in stress signaling, we studied whether ABA or other stress stimuli have an effect on the sub-cellular localization of CPK32. In a protoplast transient expression assay, we found that CPK32-GFP is localized to the plasma membrane, and upon treatment with ABA, CPK32 becomes cytosolic and moves into the nucleus. Most CPKs including CPK32 possess a myristoylation and palmitoylation signal at the N-terminus and are predicted to have membrane association (Cheng et al., 2002). In a proteomic study using isotopic radio-lalelled measurements involving several membrane predicted proteins, CPK32 has been predicted to have a trans-membrane domain (Nelson et al., 2006). Also, the CPK32 protein sequence contains an N-terminal Gly-2 and Cys-4 and Cys-5, which are key residues important for proper membrane association of several proteins through myristoylation and palmitoylation respectively. Martin and Busconi, 2000, have experimentally shown that membrane associated OsCPK2 requires myristoylation and palmitoylation for proper membrane localization and mutations in these residues lead to its cytosolic localization. Several other CPKs such as AtCPK2 (Lu

and Hrabak, 2002), a tomato CRK, LeCRK1 (leclercq et al., 2005), require myristoylation and/or palmitoylation for membrane association (Hemsley and Grierson, 2008), whereas CBL1 requires palmitoylation for trafficking between the plasma membrane and ER (Batistic et al., 2008). Since palmitoylation is a reversible process, in many proteins it is important for shuttling of membrane associated proteins between the cytosol and membranes (Wedengaertner et al., 1995) as well as for protein-protein interactions (Resh, 2006). The G protein subunit α is a well studied example of a protein trafficks between the plasma membrane and the cytosol through palmitoylation and depalmitoylation-dependent interaction with G protein subunits βγ (Resh, 2006). Palmitoylation is also important for regulating the kinase activity of G protein coupled receptor kinase, GRK6, which shows ten times more activity in the palmitoylated form compared with the non-palmitoylated form (Stoffel et al., 1998). Here, we suggest that palmitoylation is an important post translational modification for the function of CPK32 in stress responses. Interestingly, the palmitoylation mutant of CPK32 (C4A/C5A) shows a cytosolic and nuclear localization similar to the one found upon ABA treatment. In *Trypanosoma cruzi*, the palmitoylation of a flagellar Ca binding protein (FCaBP) is regulated by Ca^{2+} (Wingard et al., 2008), which suggest that palmitoylation is not only a post-translation lipid modification required for membrane association but is also important for regulating protein function. It is possible that CPK32 might be undergoing de-palmitoylation in response to ABA, dissociate from the plasma membrane, and move into the nucleus where it phosphorylates substrates that are components of ABA dependent stress signaling.

Also, in our study, we did not see any significant change in the expression of several ABA response genes due to CPK32. ABA signaling involves several regulators which act at the transcriptional, translational as well as at post translational levels (Nelson and Assman, 2007). However, being a protein kinase CPK32 might be involved in regulating ABA response genes such as ABFs that are positive regulators of ABA signaling by posttranslational phosphorylation.

To understand the possible signaling function of CPK32, we studied its effect on the expression of RD29A. RD29A is a key regulator in abiotic stress signaling including drought and ABA responses (Shinozaki and Yamaguchi-Shinozaki, 2007). In protoplast transient expression assays, when CPK32 was co-expressed with RD29A, CPK32 repressed induction of RD29A by ABA (Fig 2.13). The myristoylation and palmitoylation double mutant of CPK32 (C2A/C4A/C5A) was unable to produce this repression response. As mentioned earlier the mutation of myristoylation and palmitoylation residues in CPK32 leads to its cytosolic and nuclear localization. It is therefore possible that these post translational protein modifications are somehow required for the signaling function of CPK32.

CONCLUSION

Here we report a comprehensive approach to understand the function of CPK32, one of the 34 CPKs from Arabidopsis. In order to assign a function to CPK32 we first studied the expression pattern of this protein using both promoter analysis as well RT-PCR analysis. The regulation of CPK32, as indicated by promoter studies and transcript regulation by many abiotic and biotic stresses is consistent with the over-representation of stress response elements in the CPK32 promoter. We characterized the growth phenotypes of transgenic lines with altered CPK32 expression in order to understand its function. Based on these growth phenotypes we propose that CPK32 might act a negative regulator of ABA signaling. In addition to ABA and salt, CPK32 was also shown to affect other stress response pathways such as drought, likely independent of ABA. In sub-cellular localization studies we found that CPK32 is localized to the plasma membrane and upon ABA treatment moves into the nucleus. How this movement is carried out remains to be elucidated. However, our data indicates that post translational modifications such as myristoylation and palmitoylation are required for membrane targeting of CPK32, since mutations in these modification sites leads to cytoplasmic and nuclear localization. What role CPK32 plays after its movement to the nucleus and whether it activates proteins that are directly or indirectly involved in stress signaling by phosphorylation, is an interesting question that remains unanswered. Overall, our data indicate that CPK32 plays an important role in multiple stress response pathways.

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Appendix A

Up-regulation of the CPK32 promoter in leaves by stress stimuli

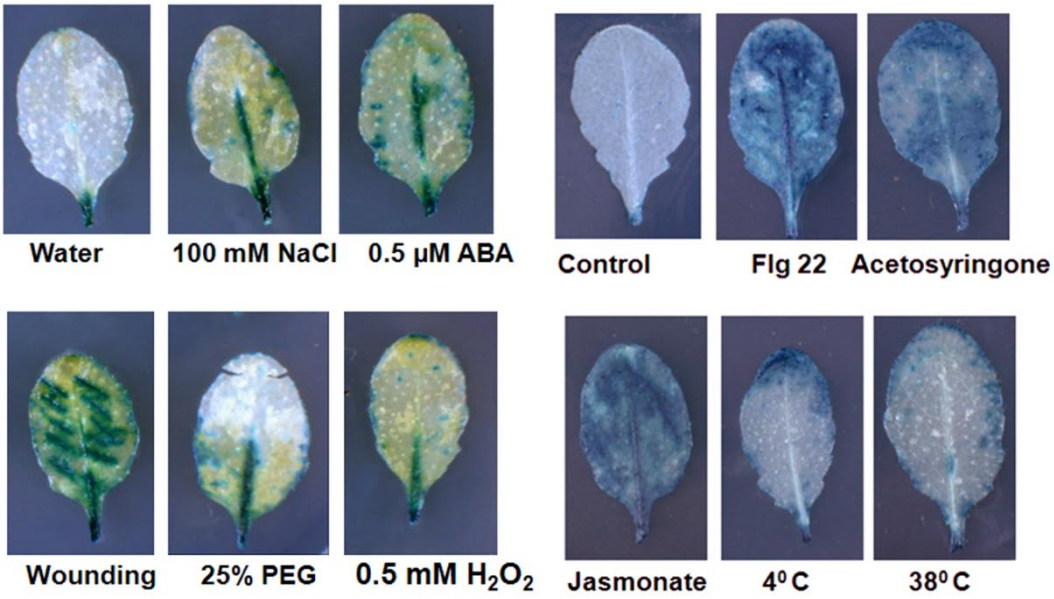

Figure A.1. Expression of the CPK32 promoter is induced in leaves after stress stimuli. Leaves from plants expressing pCPK32 GUS were treated with abiotic and biotic stress stimuli and then used for GUS staining. Leaves were photographed after de-staining in ethanol. Entire experiment was repeated three times. The following 2 h stress treatments were used: ABA = 10 µM; salt = 100 mM NaCl; PEG = 10%; H_2O_2; heat = 38^0C; cold = 4^0C; Jasmonate = 100 µM methyl jasmonate; Flg 22 = 1 µM bacterial flagellum peptide; and, ACE = 100 µM acetosyringone. Wounding was performed by making small cuts on leaf lamina without damaging the midrib, as described in Materials and Methods.

Appendix B

Identification of proteins that might interact with CPK32

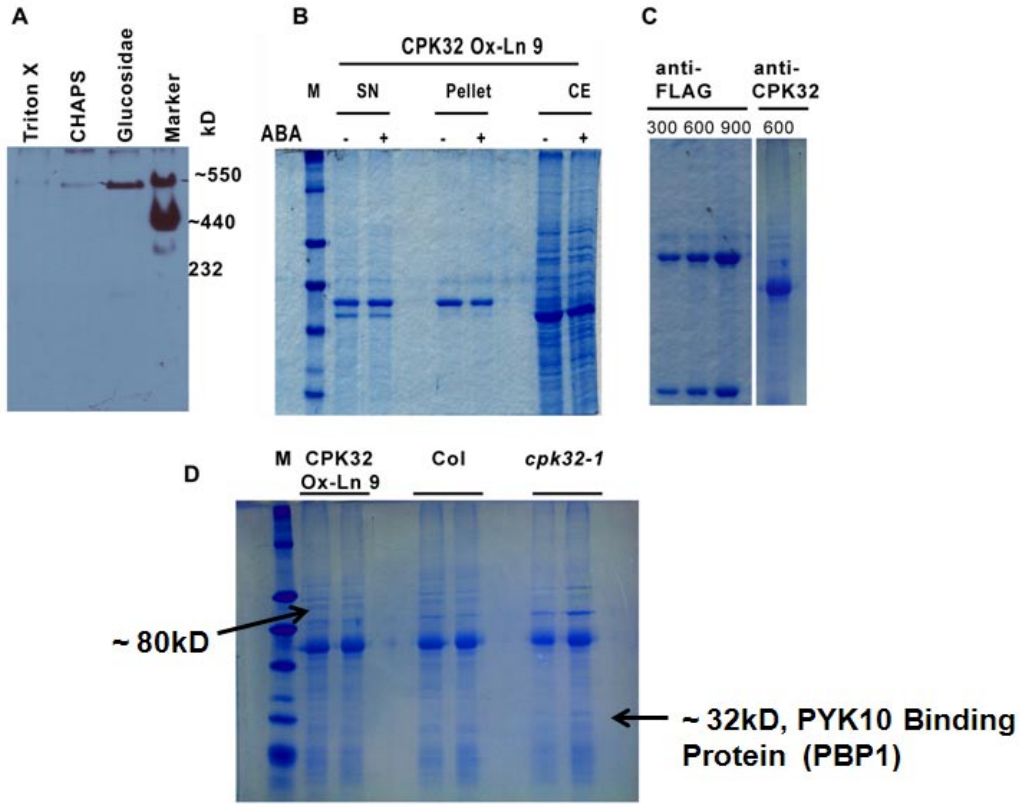

Figure B.1. Identification of proteins that might interact with CPK32. A, Western blot of proteins isolated from CPK32 overexpression plants Ox-Ln9 using Triton-X 100, CHAPS and glucosisase detergent and separated using Blue Native gel electrophoresis. B, SDS-PAGE gels stained with Colloidal Commassie Blue. Proteins were immunoprecipitated using anti-FLAG antibody and eluted in 2X SDS loading dye prior to separation. M; Protein Marker, SN; Supernatant, Pellet; re-extracted pellet, CE; crude extract. C, protein amount titration using 300, 600, and 900 µg total protein for immunoprecipitation suing either anti-FLAG or anti-CPK32 antibody.

Appendix C

Identification of proteins that might interact with CPK32

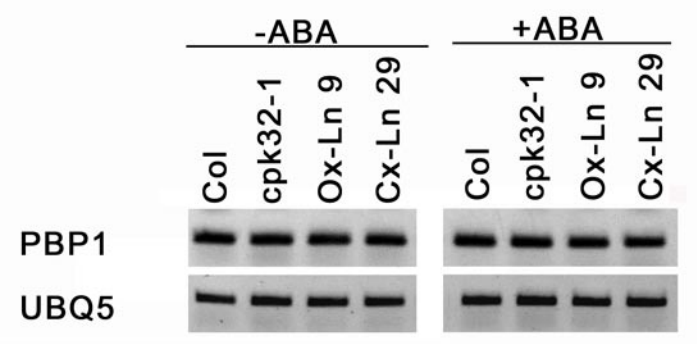

Figure C.1. CPK32 does not affect transcript levels of PBP1. Semi-quantitative RT-PCR was used to determine transcript abundance of PBP1 in CPK32 overexpression and mutant plants. UBQ5 was used as a amplification control. For ABA treatment, seedling of Col, , *cpk32-1*, Ox-Ln9, and Cx-Ln29 were grown on agar plates and then treated with water or water with 50 μM ABA for 4 h.

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Fig #2: Schematic Diagrams of the Structures of members of the CDPK/SnRK family of protein kinases.

Thank you

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