CHAPTER I

INTRODUCTION

Soil salinity is a major constraint to food production because it limits crop yield and restricts use of land previously uncultivated and is becoming a serious problem in several parts of the world. A saline area ($4x10^8$–$9x10^8$ ha) was estimated to be three times larger than land currently used for agriculture (Binzel and Reuveni, 1994). In saline soil, the low water potential directly affects efficiency of water use, because plants need to develop more negative water potential to maintain a downhill gradient of water potential between the soil and the plant. Moreover, salts in saline soil directly damage the plant cells, tissues and organelles. Salt sensitive species are susceptible to injury by salt stress, resulting in loss of leaf expansion and chlorophyll synthesis prior to plant death (Cha-um *et al.*, 2006). Plants have developed multiple strategies to achieve salt tolerance. During the process of salt adaptation, the expression of many salt-stress related genes was induced, and the synthesis of a variety of proteins was increased (Xujun *et al.*, 2005). Salt stress tolerance requires a complex interplay of signaling cascades (Xiong *et al.*, 2002), and is determined by multiple factors consisting of effectors and regulators (Hasegawa *et al.*, 2000).

Figure 1.1 shows the generic signal transduction pathway that starts with signal perception, followed by the generation of second messengers. Signal transduction requires the proper spatial and temporal coordination of all signaling molecules. Thus, there are certain molecules that participate on modification, delivery, or assembly of, signaling components, but do not directly relay the signal (Xiong *et al.*, 2002) as shown in Figure 1.1. Because different signals often elicit distinct and specific cellular responses

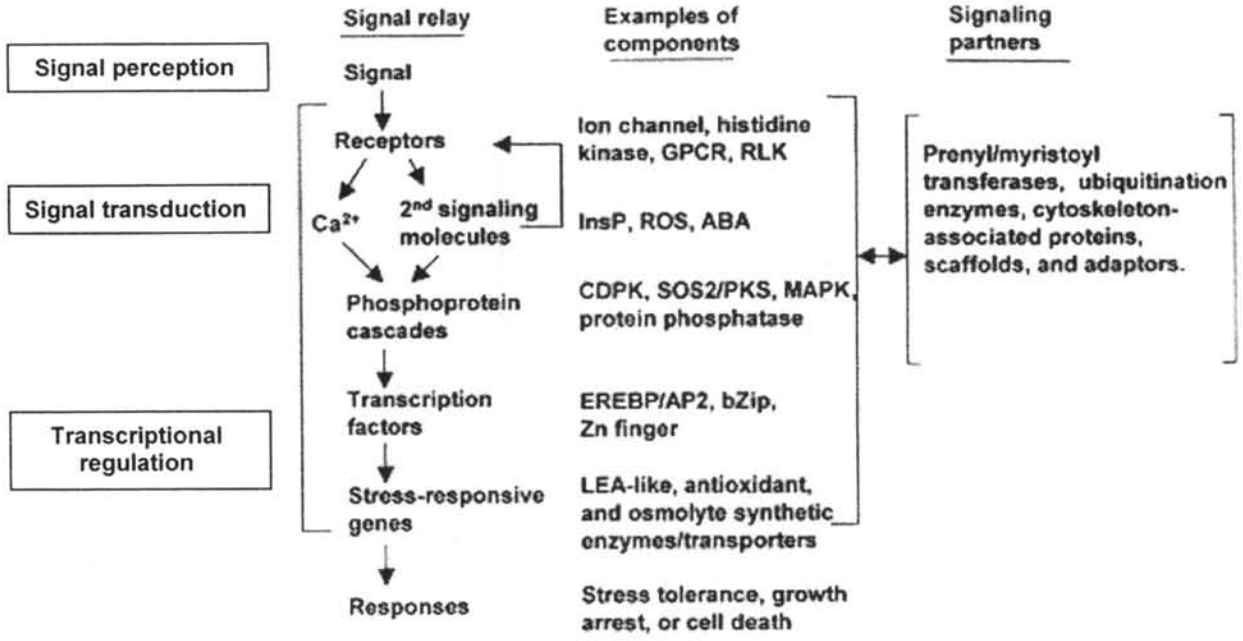


Figure 1.1 A generic pathway of signal perception and signal transduction in Plants. Examples of signaling components in each of the steps are shown. Secondary signaling molecules can cause receptor-mediated Ca^{2+} release (indicated with a feedback arrow). Examples of signaling partners that modulate the main pathway are also shown. These partners can be regulated by the main pathway. Signaling can also bypass Ca^{2+} or secondary signaling molecules in early signaling steps. GPCR, G-protein coupled receptor; InsP, inositol polyphosphates; RLK, receptor-like kinase; ROS, reactive oxygen species; ABA, abscisic acid; CDPK, Ca^{2+}-dependent protein kinases; SOS2/PKS, Salt Overly Sensitive 2/Protein kinases; MAPK, mitogenactivated protein kinase; EREBP/AP2, ethylene-responsive element binding proteins/APETALA2; bZIP, basic leucine zipper; Zn finger, Zinc finger; and LEA-like, late embryogenesis–abundant. (Xiong *et al.*, 2002).

it is important to determine how plants sense various signals and produce an appropriate response. Salt stress signal transduction is made up of multiple pathways that consist of ionic and osmotic homeostasis signaling pathways, detoxification response pathways, pathways for growth regulation and calcium signaling pathway (Zhu, 2001). Components of signaling pathways operating in salt stress-affected cells and interactions between these pathways are main research subjects in many laboratories.

Calcium signaling

Calcium signaling plays an important role in plants for coordinating a wide range of developmental processes and responses to hormonal and environmental signals such as salinity, cold, light, drought, symbiotic and pathogenic elicators. It appears that different stimuli elicit specific calcium signatures, generated by altering the kinetics, magnitude, and cellular source of the influx (Malhó *et al.*, 1998; Allen *et al.*, 2000, 2001; Evans *et al.*, 2001; Rudd and Franklin-Tong, 2001). Research during the last two decades has clearly established that Ca^{2+} acts as an intracellular signal to specific responses (Reddy, 2001). At low resting levels, the concentration of Ca^{2+} in the cytoplasm of plants cells is maintained in the nanomolar range (100-200 nM) by being actively pumped into intracellular compartments and extracellular spaces with $[Ca^{2+}]$ is in the millimolar range (1-10 mM) as shown in Figure 1.2 (Reddy, 2001). The export of Ca^{2+} ions from the cytosol to the extracellular space or into intracellular organelles is achieved by ATP-driven Ca^{2+}-pumps and antiporters (Vetter and Leclerc, 2003).

Different stimuli elicit Ca^{2+} transients which are distinct in their subcellular localization, amplitude, duration, frequency of oscillation and mode of spatial

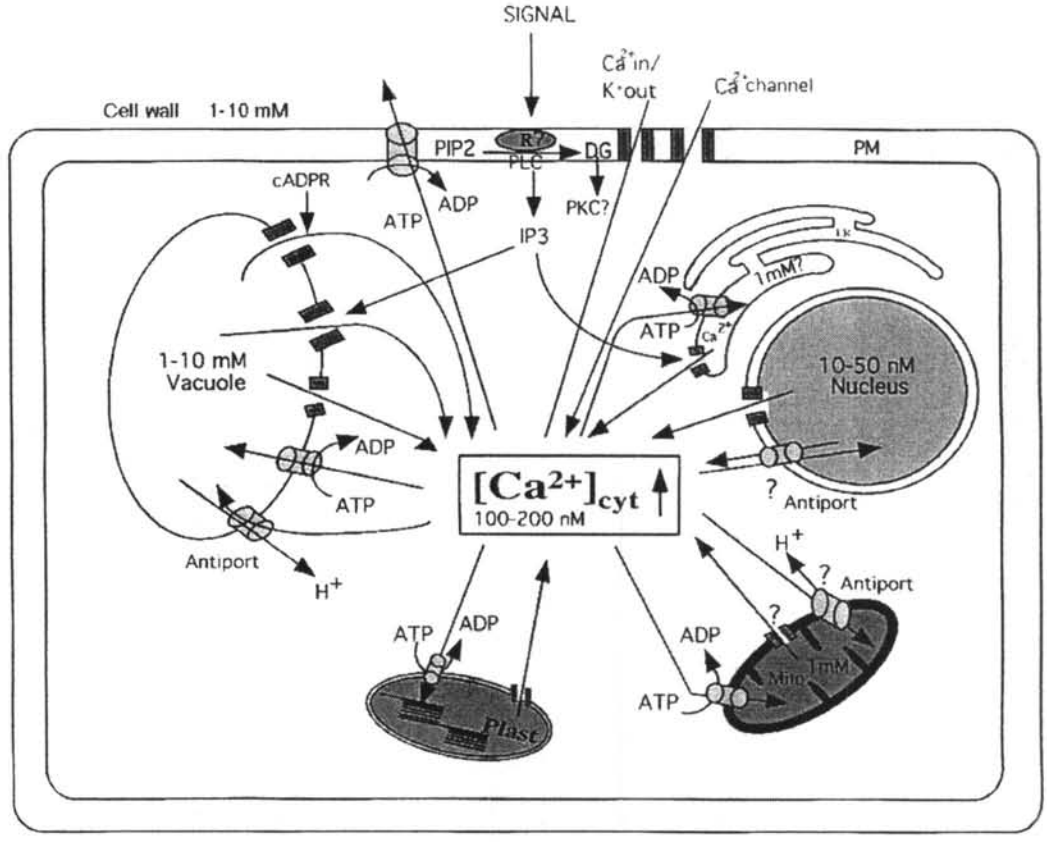


Figure 1.2 Schematic diagram illustrating the mechanisms by which plant cells elevate $[Ca^{2+}]_{cyt}$ in response to various signals and restore Ca^{2+} concentration to resting level. Ca^{2+} channels are shown in red, whereas Ca^{2+} ATPases and antiporters are indicated in yellow. Arrows indicate the direction of Ca^{2+} flow across the plasma membrane, and into and out of cellular organelles (vacuole, plastids, mitochondria, endoplasmic reticulum and nucleus). The estimated concentration of resting levels of Ca^{2+} in different organelles is indicated. Question marks indicate the lack of evidence. $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+}; PLC, phospholipase C; R, receptor, cADPR, cyclic ADP ribose, PIP_2, phosphotidyl ino sitol-4,5-bisphosphate, DG, diacylglycerol, PKC, protein kinase C, IP_3, inositol-1,4,5-trisphosphate; ER, endoplasmic reticulum; Mt, mitochondria; Plast, plastids; PM, plasma membrane (Reddy, 2001).

propagation (Snedden and Fromm, 2001). These properties are highly coordinated and regulated by the spatial distribution of Ca^{2+}-release channels throughout the cell. The influx of Ca^{2+} ions are generated by voltage- and ligand-gated Ca^{2+}-permeable channels on the plasma membrane. In addition, several intracellular organelles function as Ca^{2+} stores, which can release Ca^{2+} upon stimulation by, for instance, inositol-1, 4, 5-trisphosphate (IP_3) or cyclic ADP-ribose (cADPR). The endoplasmic reticulum (ER) is a major Ca^{2+} stores, but mitochondria and the nucleus also participate actively in the release of Ca^{2+} through the IP_3-receptor. An important feature of the role of Ca^{2+} as a signal is the presence of repetitive Ca^{2+} transients. These transients may be generated both by first-round second messengers and by signaling molecules such as ABA that may themselves be produced as a result of cascades of early Ca^{2+} signals as shown in Figure 1.3. These rounds of signals may have quite different signaling consequences and, therefore, physiological meaning. (Xiong *et al.*, 2002)

The EF-hand motif

The approximately 100-fold increase in free Ca^{2+} concentration upon stimulation of a cell allows Ca^{2+}-binding proteins to bind Ca^{2+} ions. Several hundred Ca^{2+}-binding proteins have been identified and most of them share a common Ca^{2+} binding motif. This motif comprises about 30 amino acids and consists of a helix-loop-helix where the two helices are arranged similar to the extended thumb and index finger of a hand: it is commonly called the EF-hand motif as shown in Figure 1.4. In almost all Ca^{2+}-binding proteins two EF-hand motifs are in close proximity forming an EF-hand pair. This consists of four helices arranged in the form of a twisted four helix bundle.

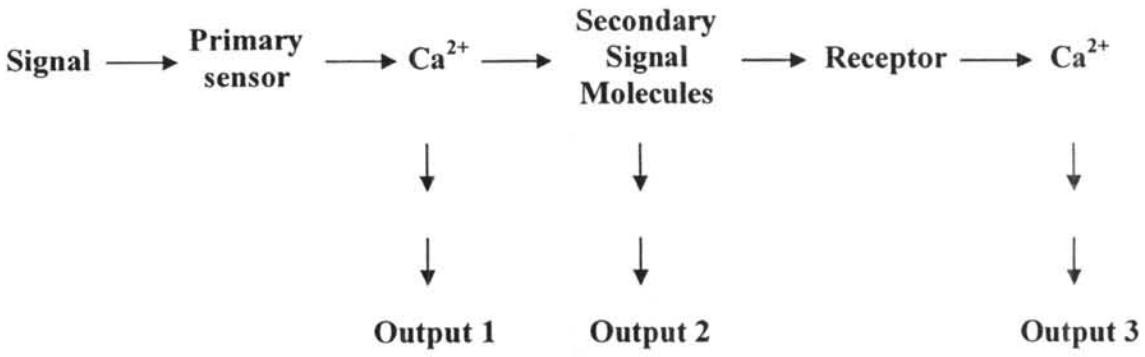
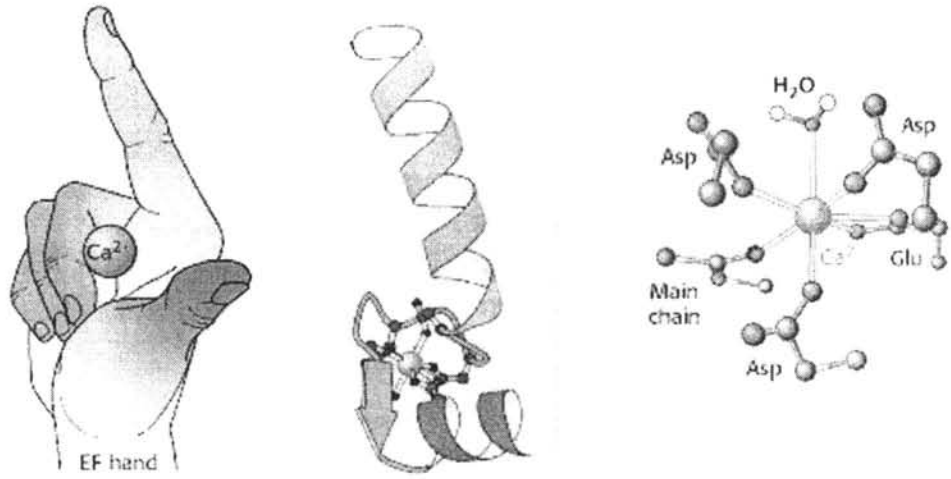


Figure 1.3 Repetitive Ca^{2+} Transients upon the Perception of a Primary Signal. The primary increase in cytosolic Ca^{2+} facilitates the generation of secondary signaling molecules, which stimulate a second round of transient Ca^{2+} increases, both locally and globally. These second Ca^{2+} transients may feedback regulate each of the previous steps (not shown). Ca^{2+} transients from different sources may have different biological significance and result in different outputs, as shown. Secondary signaling molecules such as ROS can also directly regulate signal transduction without Ca^{2+} (Output 2) (Xiong *et al.*, 2002).



A) B) C)

Figure 1.4 The tertiary structure of EF hand motif. (A) A symbolic representation of the EF-hand motif. The helix E winds down the index finger, where as the helix F winds up the thumb of a right hand, (B) helix-loop-helix; E helix, loop and F helix are displayed in yellow, orange and blue respectively, (C) The geometry of Ca^{2+} ligands that interact with six amino acids and a water molecule within the loop.

The EF hands have the canonical 12-residue Ca^{2+} binding loop. Ca^{2+} is bound in a pentagonal bipyramidal geometry with seven sites of coordination occurring through interactions with six amino acids, those in position 1, 3, 5, 7, 9 and 12 (alternatively called +X, +Y, +Z, -Y, -X, and -Z) within the region designated as +X*+Y*+Z*-Y*-X**-Z, in which * represents an intervening residue as shown in Figure 1.5. All of these amino acids interact with Ca^{2+} through side chain oxygens, except residue seven, which acts through its main chain oxygen. Three ligands for Ca^{2+} coordination are provided by carboxylate oxygens from residues 1 (+X), 3 (+Y) and 5 (+Z), one from a carbonyl oxygen from residue 7 (-Y), and two from carboxylate oxygens in residue 12 (-Z). The seventh ligands is provided either by a carboxylate side chain from residue 9 (-X), or from a water molecule received via the side chain or carbonyl oxygen of residue 9. Thus, there are strong preferences for specific amino acids within the Ca^{2+}-binding loop. The X position is almost exclusively filled with aspartate (D); Y is usually aspartate (D) or asparagines (N); Z is aspartate (D), asparagine (N), or serine (S); the -Y position tolerates a variety of amino acids; -X also varies, but is usually aspartate (D), asparagine (N), or serine (S); -Z, which contributes two coordination sites, is nearly invariably glutamate (E). Glycine (G) at position 6 is highly conserved and is thought to provide the ability for a sharp turn within the loop. Finally, position 8 is most often isoleucine (I), which can form hydrogen bonds with the other EF loop in a pair. The cysteine (C) residue in position 7 of the first EF hand is common among plant CaMs (Zielinski, 1998), but uncommon in nonplant CaMs.

The E helix generally starts with a glutamate (E); both the E and F helices flanking the Ca^{2+}-binding loop are generally each 9 amino acids long. There is a regular

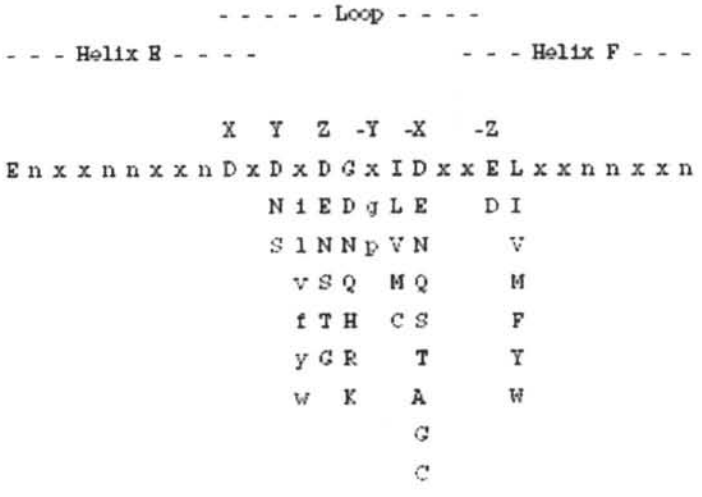


Figure 1.5 Consensus sequence for EF-hands. (Harmon, 2003). "n" represents hydrophobic amino acids and "x" represents any amino acid

distribution of hydrophobic amino acids in the E helices with a pattern of 'nxxnnxxn' where 'n' represents hydrophobic amino acids and 'x' represents any amino acid. In CaMs, the pattern is similar for the F helices of hands 1 and 3, but diverges slightly in hands 2 and 4. The Ca^{2+}-binding domains are numbered I through IV, beginning from the amino-terminus.

Ca^{2+}-binding proteins

Calcium elevation is sensed by several Ca^{2+}-binding proteins and decoded via Ca^{2+}-dependent conformational changes in these sensor polypeptides and interacting target proteins. A large number of Ca^{2+} sensors can be grouped into four major classes as shown in Figure 1.6. These include (A) Ca^{2+}-dependent protein kinase (CPK) that contains CaM-like Ca^{2+} binding domains and a kinase domain in a single protein. Each individual CPK protein is expected to detect the changes in the Ca^{2+} parameters and translate these changes into the regulation of a protein kinase activity (Roberts and Harmon, 1992), (B) Calmodulin (CaM) which contains four EF-hand domains but have no enzymatic activity themselves and function by interacting with their target proteins (Zielinski, 1998), (C) other EF-hand motif-containing Ca^{2+}-binding proteins and Calcineurin B-like (CBL) protein that are similar to both the regulatory B subunit of calcineurin and the neuronal Ca^{2+} sensor (NCS) in animals (Klee *et al.*, 1998) and (D) Ca^{2+}-binding proteins without EF-hand motifs.

Members of the first three classes of Ca^{2+} sensors contain the EF-hand helix-loop-helix motifs that bind to Ca^{2+} with high affinity (Roberts and Harmon, 1992). However, different Ca^{2+}-binding proteins differ in the number of EF hand motifs and their affinity

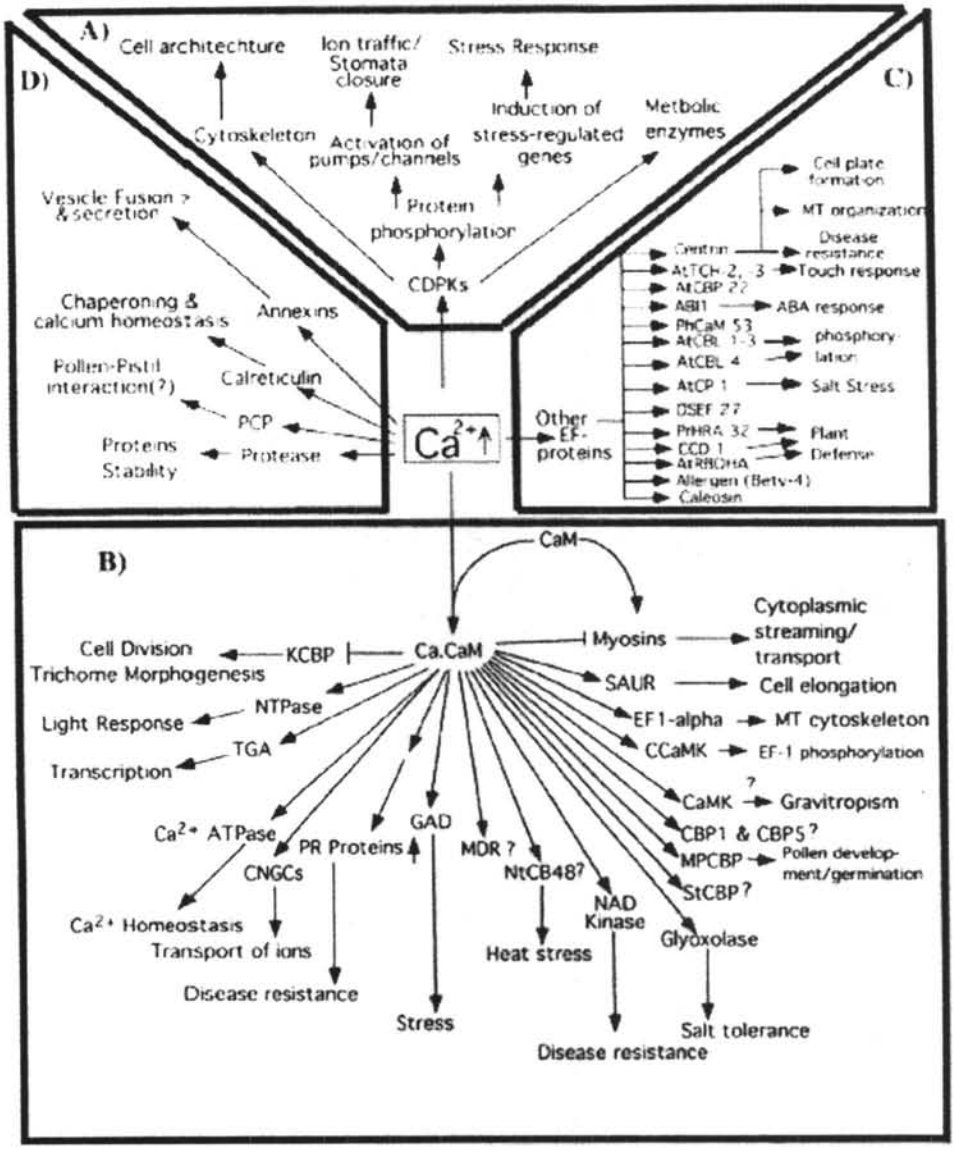


Figure 1.6 Ca^{2+} sensing proteins and their functions in plants. Four major groups of Ca^{2+} sensors (indicated in four boxes) have been described in plants: (A) Ca^{2+}-dependent protein kinase (CDPK), (B) Calmodulin (CaM), (C) other EF-hand motif-containing Ca^{2+}-binding proteins and Calcineurin B-like (CBL) protein, (D) Ca^{2+}-binding proteins without EF-hand motifs (Reddy, 2001).

to Ca^{2+} with dissociating constants (K_ds) ranging from 10^{-5} to 10^{-9} M. Binding of Ca^{2+} to a Ca^{2+} sensor causes a conformational change in the sensor resulting in modulation of its activity or its ability to interact with and modulate function/activity of other proteins (Reddy, 2001).

Calmodulin

Calmodulin is probably the most well characterized Ca^{2+} sensors among these groups of protein. It is a small (148 residues, 17 kDa), highly conserved, soluble, intracellular Ca^{2+}-binding protein ubiquitously found in animals, plants, fungi and protozoa, and is regarded as a major transducer of Ca^{2+} signals in mammalian cells. It has four EF-hands that function in pairs. Many proteins involved in Ca^{2+} signal transduction alter their activity in response to changes in free Ca^{2+} levels, but are themselves not able to bind Ca^{2+} ions. Some of these proteins utilize CaM as a sensor and mediator of the initial Ca^{2+} signal. Calmodulin is multifunctional protein because of its ability to interact and regulate the activity of a number of other proteins as shown in Figure 1.7. CaM relays the Ca^{2+} signal by binding free Ca^{2+} ions to its C- and N-terminal EF-hand pairs, which causes a conformational change and enables Ca^{2+}/CaM to bind to specific CaM-binding domains. The binding of Ca^{2+}/CaM to its target proteins alters their activity in a calcium dependent manner.

Ca^{2+}-bound-calmodulin-mediated signal transduction in plants is shown in Figure 1.8. Biotic and abiotic signals are perceived by receptors, resulting, in some cases, in transient changes in Ca^{2+} concentrations in the cytosol and/or organelles (e.g. nucleus). Increases in free Ca^{2+} concentrations originating from either extracellular pools or

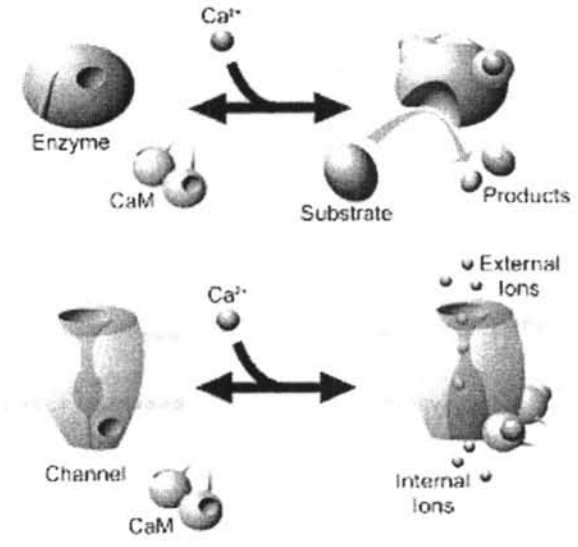


Figure 1.7 Calmodulin acts as a multifunctional protein.

intracellular stores are capable of binding to Ca^{2+}-modulated proteins including calmodulin and calmodulin-related proteins. Structural modulations of these proteins enable them to interact with numerous cellular targets that control a multitude of cellular functions, such as metabolism, ion balance, the cytoskeleton and protein modifications. In addition, Ca^{2+} and calmodulin might also regulate the expression of genes by complex signaling cascades or by direct binding to transcription factors. Rapid changes in cellular functions result from direct interactions of calmodulin and calmodulin-related proteins with their targets (within seconds to minutes) while slower responses require gene transcription, RNA processing and protein synthesis (variable times from minutes to days).

The EF hands in CaM are organized into two distinct globular domains, each of which contains one pair of EF hands. Each pair of EF hands is considered the basic functional unit. Pairing of EF hands is thought to stabilize the protein and increase its affinity toward Ca^{2+}. Although each globular domain binds Ca^{2+} and undergoes conformational changes independently, the two domains act in concert to bind target proteins. Upon increase of Ca^{2+} concentration to submicromolar or low micromolar levels, all CaM molecules are activated. Cooperative binding is required for this "on/off" mechanism to function efficiently. The cooperatively of Ca^{2+} binding ensures that full activation of the CaM occurs in a narrow region of calcium concentration during a signaling event.

The selectivity of CaM toward Ca^{2+} also is an important factor in effective transduction of the Ca^{2+} signal. CaMs bind Ca^{2+} selectively in the presence of high concentrations of Mg^{2+} and monovalent cations in the cell. The cation selectivity is achieved

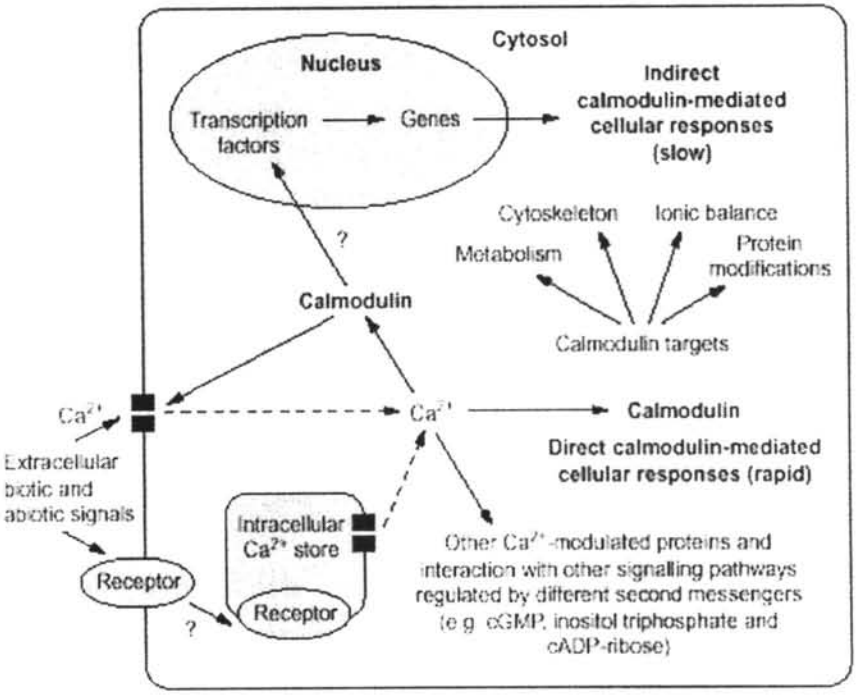


Figure 1.8 The Ca^{2+}-bound-calmodulin-mediated signal transduction in plants. Broken arrows denote Ca^{2+} fluxes from extracellular or intracellular stores, and question marks signify unknown signal transduction intermediates.

by optimizations in the structure folds of the binding loop. For example, discrimination between Ca^{2+} and Mg^{2+} is accomplished through reduction in the size of the binding loop. Binding of Mg^{2+} ions would collapse the EF-hand loop, thereby reducing the distance between negatively charged side chains and destabilizing the CaM- Mg^{2+} complex (Falke *et al.*, 1994). Even small changes in the chemical properties of the Ca^{2+} binding loop (e.g., Glu-12→Gln) can drastically reduce the binding affinity to Ca^{2+} (Beckingham, 1991; Haiech *et al.*, 1991). The Glu-12→Gln mutation changes the carboxylate side chain into carboxylamide, which removes the oxygen ligand for Ca^{2+}. Together, structural analyses in combination with site-directed mutagenesis established that CaMs (and other EF hand–containing proteins, including CBLs) have evolved as highly specific Ca^{2+} sensors (Luan *et al.*, 2002).

The overall structure of Ca^{2+}/CaM is dominated by two EF-hand pairs forming the C- and N- terminal lobes and a long α-helix connecting the two lobes. In vertebrate CaM, the two EF-hand pairs share 48% sequence identity and 75% sequence similarity and the peptide backbone of the two lobes can be superimposed with a mean square derivation of ~0.7 A° (Vetter and Leclerc, 2003). Structural analysis of the Ca^{2+}-free and Ca^{2+}-bound states of CaM proteins reveals the conformational changes induced by Ca^{2+} binding as shown in Figure 1.9. In the Ca^{2+}-free state, CaM adopts a closed conformation. Ca^{2+} binding triggers a conformational change, and the protein adopts an open conformation with nearly perpendicular interhelical angles between the globular domains. This open conformation exposes a hydrophobic surface within each globular domain and permits the binding of protein targets (Babu *et al.*, 1988; Kuboniwa *et al.*, 1995, Zhang *et al.*, 1995).

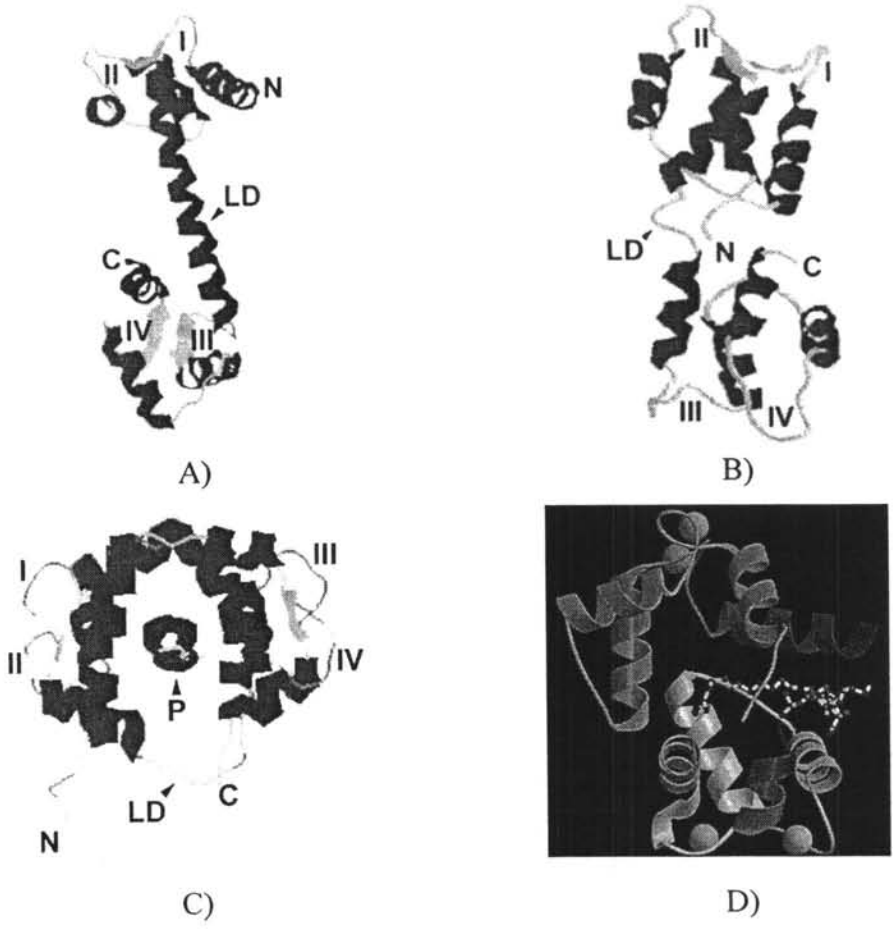


Figure 1.9 Ribbon presentations of Calmodulin. (A) Ca^{2+}/Calmodulin (CaM) determined by X-ray crystallography, (B) globular domain of CaM (apo-CaM) determined by NMR spectroscopy, (C) and (D) The direction of Ca^{2+}/CaM-target peptide interaction; Peptide binding causes disruption of the flexible tether, bringing the globular domains closer to form a channel around the peptide. The majority of contacts between Ca^{2+}-CaM and target peptide are nonspecific van der Waals bonds made by residues in the hydrophobic surfaces. For (A) to (C), I-IV, Ca^{2+}-binding loops in the EF-hands; N, amino- termini of the CaM; C, carboxy-termini of the CaM; LD, central linker domain and P, target peptide. The helices, loop and b-sheet are colored in red, blue and yellow respectively.

Ca^{2+}-CaM binds and regulates the activity of a wide range of proteins that are not necessarily related in structure. How can Ca^{2+}-CaMs bind to so many different proteins? More specifically, the plasticity of the Ca^{2+}-CaM structure must accommodate the variation in both the molecular size and the composition of the target proteins. This issue has been addressed by structural analyses of Ca^{2+}-CaM and target-bound Ca^{2+}-CaM. Figure 1.8C shows that the two globular domains of Ca^{2+}-CaM are connected by a flexible tether that can accommodate peptides of varying sizes. The binding of CaM-binding peptides is largely driven by hydrophobic interactions between hydrophobic anchor residues of the peptide with the hydrophobic surface cavities of CaM. Methionine residues, unusually abundant in CaM, play a particularly important role in the binding of target peptides. The methionine side chains are very flexible and the sulfur atom has a larger polarizability than carbon, resulting in stronger van der Waals interactions. The hydrophobic patches of each lobe are surrounded by several charged residues, creating charged binding channel outlets. The C-terminal end of the peptide-binding channel has a negatively charged rim, whereas the N-terminal hydrophobic patch has clusters of negatively and positively charged residues. This charge distribution on the molecular surface contributes to peptide binding via electrostatic interactions and determines the relative binding orientation of CaM-binding domain peptides. Basic residues at the N-terminus of the peptide form salt bridges with acidic residues surrounding the peptide-binding channel of the C-terminal lobe of CaM. Together, the structures of CaM illustrate how this class of proteins can function as extremely efficient Ca^{2+} sensors and on/off switches, allowing them to transduce Ca^{2+} signals with high efficiency and accuracy. Different affinities for Ca^{2+}-CaM interactions with specific target proteins may be

sufficient for the differential transduction of the Ca^{2+} signal (Luan *et al.*, 2002).

In plants, there are multiple *CaM* genes that code for either identical proteins or proteins containing a few conservative changes. These small changes in amino acid composition of CaM isoforms may contribute to differential interaction of each CaM isoform with target proteins. The striking example for differential regulation of CaMs comes from the studies with soybean CaM isoforms. In soybean there are five CaM isoforms (SCaM1 to -5). SCaM1, -2 and -3 are highly conserved compared to other plant CaM isoforms including Arabidopsis CaM isoforms whereas SCaM4 and -5 are divergent and showed differences in 32 amino acids with the conserved group (Lee *et al.*, 1995). Surprisingly, these divergent CaM isoforms are specifically induced by fungal elicitors or pathogen (Heo *et al.*, 1999). These results provided evidence for the differential regulation of CaM isoforms in plants. Soybean isoforms show differences in their relative abundance *in vivo*. The conserved isoforms are relatively abundant in their expression compared to divergent forms. All CaM isoforms activate phosphodiesterase (PDE) but differ in their activation of NAD kinase, calcineurin and nitricoxide synthase indicating Ca^{2+}/CaM specificity between CaM isoform s and target proteins (Lee *et al.*, 1997). Differential regulation of enzymes by soybean divergent and conserved CaM isoforms has also been reported (Lee *et al.*, 2000). Although SCaM isoforms show similar patterns in protein blot overlay assays, they differ in their relative affinity in interacting with CaM binding proteins (Lee *et al.*, 1999). In another samples, two divergent CaM isoforms that are found in Arabidopsis do not interact with proteins that bind to conserved CaM isoforms (Kohler *et al.*, 2000). These studies suggest that conserved and divergent CaM isoforms may interact with different target proteins.

Recent studies on CaM genes expression in response to different stimuli indicate that different CaM isoforms are involved in mediating a specific signal (Zielinski, 1998). There is considerable evidence to indicate that CaM genes are differentially expressed in response to different stimuli. Three of the six Arabidopsis *Cam* genes (*Cam*1, -2 and -3) are inducible by touch stimulation (Zielinski, 1998) indicating the presence of different *cis*-regulatory elements in their promoters. In potato, only one of the eight CaM isoforms (PCaM1) is inducible by touch (Takezawa *et al.*, 1995). The presence of multiple CaM isoforms adds further complexity to the Ca^{2+} mediated network. Even though a large family of genes encoding CaM and closely related proteins from several plants has been identified, with the exception of Arabidopsis, families of genes encoding CaM and related proteins have not been extensively conducted in a whole-genome scale. In Arabidopsis, McCormack and Braam have characterized members of Groups IV and V members from the 250 EF-hand encoding genes identified in the Arabidopsis genome. Seven loci are defined as *Cam* genes and 50 additional genes are CaM-like (*CML*) genes, encoding proteins composed mostly of EF-hand Ca^{2+}-binding motifs (McCormack and Braam, 2003, McCormack *et al.* 2005). The high complexity of the CaM and related calcium sensor proteins in Arabidopsis suggests their important and diverse roles of calcium signaling.

Abscisic acid (ABA)

The plant hormone abscisic acid (ABA) regulates many agronomically important aspects of plant development, including the synthesis of seed storage proteins and lipids, the promotion of seed desiccation tolerance and dormancy, and the inhibition of the phase

transitions from embryonic to germinative growth and from vegetative to reproductive growth. Structure of ABA is shown in Figure 1.10. In addition, ABA mediates some aspects of physiological responses to environmental stresses such as drought- or osmotica-induced stomatal closure, the induction of tolerance of water, salt, hypoxic, and cold stress, and wound or pathogen response. A traditional distinction among these responses has been that of speed: the stomatal responses are relatively fast, occurring within minutes and involving changes in the activity of various signaling molecules and ion channels, whereas the rest is slower and requires changes in gene expression.

Salinity causes increased biosynthesis and accumulation of abscisic acid (ABA), which can modulate physiological processes in plant responses to salinity. ABA limits Na^+ and Cl^- accumulation in leaves, which appears to be the result of a restriction of root-to-shoot ion transport via a transcriptional stream because ABA is a stomatal regulator. Recently, the role of ABA in stress signal transduction has been investigated intensively and the results indicate that ABA can upregulate many stress-responsive genes (Hasegawa *et al.*, 2000; Zhu, 2002). In adult plants, ABA serves as a critical chemical messenger for stress responses. Several studies have shown that ABA accumulation is required for the development of stress tolerance in plants. However, the extent and the molecular basis of ABA involvement in stress-responsive gene expression and stress tolerance were not immediately clear.

Increased ABA levels under drought and salt stress are mainly achieved by the induction of genes coding for enzymes that catalyze ABA biosynthetic reactions. The ABA biosynthetic pathway in higher plants is understood to a great extent. Zeaxanthin epoxidase (ZEP; encoded by *ABA1* in Arabidopsis and *ABA2* in tobacco; Marin *et al.*,

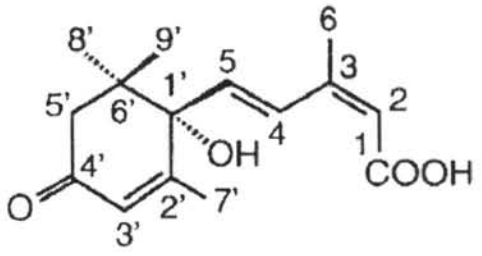


Figure 1.10 Structure of *S*-(+)-abscisic acid.

1996) catalyzes the epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Rock and Zeevaart, 1991). The 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the oxidative cleavage of 9-*cis*-neoxanthin to generate xanthoxin (Schwartz *et al.*, 1997b; Tan *et al.*, 1997). It is thought that xanthoxin is converted to ABA by a two-step reaction via ABA-aldehyde. The Arabidopsis *aba2* mutant is impaired in the first step of this reaction, and is thus unable to convert xanthoxin into ABA-aldehyde (Léon-Kloosterziel *et al.*, 1996). The Arabidopsis *aba3* mutant is defective in the last step of ABA biosynthesis, i.e., the conversion of ABA-aldehyde to ABA (Schwartz *et al.*, 1997a; Bittner *et al.*, 2001), which is catalyzed by ABA-aldehyde oxidase (AAO) as shown in Figure 1.11. Mutations in either the aldehyde oxidase apoprotein (Seo *et al.*, 2000) or molybdenum cofactor biosynthetic enzymes (e.g. molybdenum cofactor sulfurase; MCSU) would impair ABA biosynthesis and lead to ABA deficiency in plants. In this ABA biosynthetic pathway, the rate-limiting step was thought to be the oxidative cleavage of neoxanthin catalyzed by NCED (Tan *et al.*, 1997; Liotenberg *et al.*, 1999; Qin and Zeevaart, 1999; Taylor *et al.*, 2000; Thompson *et al.*, 2000). Expression studies with *ZEP*, *NCED*, *AAO3*, and *MCSU* indicated that these genes are all upregulated by drought and salt stress (Audran *et al.*, 1998; Seo *et al.*, 2000; Iuchi *et al.*, 2001; Xiong *et al.*, 2001b, 2002), although their protein levels were not examined in every case. However, the mechanisms by which drought or salt stress that upregulate ABA biosynthetic genes are not understood. Whether and how ABA regulates its own biosynthetic genes is not clear. Recent studies suggest that all of those genes (i.e., *ZEP*, *NCED*, *AAO3*, and *MCSU*) are likely regulated through a common cascade that is Ca^{2+}-dependent.

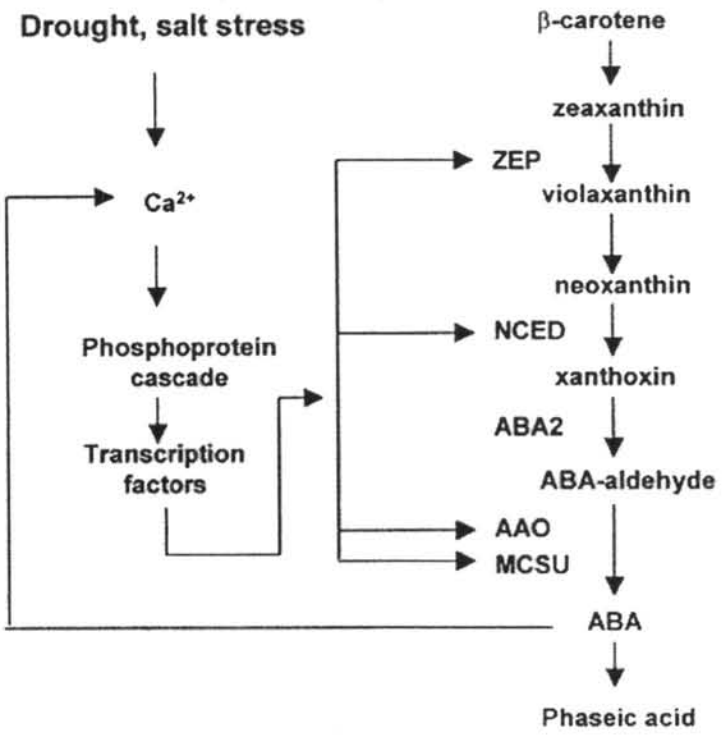


Figure 1.11 Pathway and Regulation of ABA Biosynthesis. ABA is synthesized from a C_{40} precursor β-carotene via the oxidative cleavage of neoxanthin and a two-step conversion of xanthoxin to ABA via ABA-aldehyde. Environmental stress such as drought, salt and, to a lesser extent, cold stimulates the biosynthesis and accumulation of ABA by activating genes coding for ABA biosynthetic enzymes. Stress activation of ABA biosynthetic genes is probably mediated by a Ca^{2+}-dependent phosphorelay cascade, as shown at left. In addition, ABA can feedbacks stimulate the expression of ABA biosynthetic genes, also likely through a Ca^{2+}-dependent phosphoprotein cascade. Also indicated is the breakdown of ABA to phaseic acid. AAO, ABA-aldehyde oxidase; MCSU, molybdenum cofactor sulfurase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; ZEP, zeaxanthin epoxidase (Xiong *et al.*, 2002).

There is a large overlap of ABA-, drought-, and salt-responsive gene expression in the vegetative tissues of adult plants (Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein *et al.*, 2002; Xiong *et al.*, 2002). Furthermore, characterization of stress gene promoters indicates that many gene promoters contain cis-acting elements that are separately responsible for abiotic stress and ABA. Because of their ABA-responsive promoter elem ents, these genes can be activated by stress-induced inc rease in ABA levels, as part of an ABA biosynthesis-dependent regulatory pathway. In addition, the stress-responsive elements are responsible for stress-responsive expression without the need of ABA biosynthesis, resulting in an ABA-independent regulatory pathway (Shinozaki *et al.*, 1998). However, some apparent ABA-independent pathways may require ABA for full response as a result of cross talk between ABA and stress response pathways (Knight and Knight, 2001; Xiong *et al.*, 2002; Kim *et al.*, 2003). It has been proposed that specific cross talk nodes may upregulate or downregulate the signaling strength and interaction between the different ABA-dependent and ABA-independent stress response pathways (Knight and Knight, 2001; Kim *et al.*, 2003). Calcium represents a prime candidate for such cross talk nodes because it has been shown to serve as a second messenger for both ABA and stress responses.

Rice as a model monocot system

Rice is the world's single most important food crop and a primary food for more than a third of the world's population. Production and consumption are concentrated in Asia where more than 90% of all rice is produced and consumed (Khush and Toenniessen, 1991). Rice refers to two species which are *Oryza sativa* and *Oryza*

glaberrima, native to tropical and subtropical southern and southeastern Asia and to Africa, respectively. Among the Asia domesticated rices, *Oryza sativa*, three sub-species are commonly distinguished based on geographic conditions, japonica (also called senica), indica and javanica, all of which include glutinous and non-glutinous varieties. The japonica varieties have narrow dark green leaves, medium-height tillers, and short to intermediate plant height. It is usually grown in cooler subtropics and temperate climates, such as Japan, Portugal, Spain, USSR, Italy, and France. The traditional indica rice varieties, widely grown throughout the tropics and subtropics, are tall and heavy tillering with long, narrow, light green leaves. *Javanicas* flourished in the equatorial region of Indonesia.

In addition to their adaptation to climate, the three races differ in characteristics of the grain, including the content of amylose, the elongation of the grain, the temperature at which the grain becomes gelatinous and the aroma in cooking. The contrasts which most immediately strike the non-specialist are that indica rices have longer, more slender grains which usually separate when cooked, while japonica rices have shorter, rounder and more translucent grains which quickly become slightly sticky.

Oryza sativa is an annual grass as shown in Figure 1.12, which grows best when submerged in water. It grows in upland areas, irrigated areas, rainfed lowland areas, and flood-prone areas. Rice is highly adaptable and can be grown in diverse environments. It resembles a weed, 2 to 5 feet tall, depending on the variety and depth of submersion. Rice is constantly bombarded with environmental signals, both biotic and abiotic, some of which cause stress and limit the growth and development and affect the yield and quality. *Oryza sativa* was the cereal selected to be sequenced as a priority and has gained the

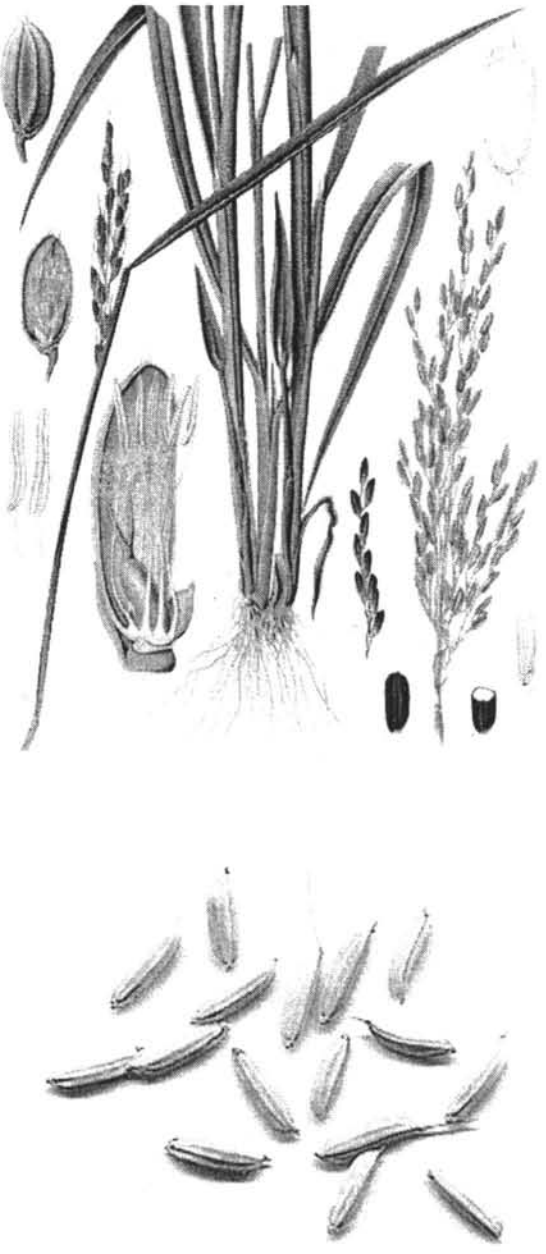


Figure 1.12 *Oryza sativa* L. (http://en.wikipedia.org/wiki/Image:Koeh-232.jpg)

status of the "model organism". Rice with its relatively small genome size (~430 Mb), ease of transformation, well developed genetics, availability of a dense physical map and molecular markers (Chen *et al.*, 2002; Wu *et al.*, 2002a), high degree of chromosomal co-linearity with other major cereal such as maize, wheat, barley and sorghum (Ohyanagi *et al.*, 2006) and together with its complete genome sequence (Sasaki *et al.*, 2005) is considered a model monocot system. It is being used to understand several fundamental problems of plant physiology, growth and developmental processes ranging from elucidation of a single gene function to whole metabolic pathway engineering. In addition, rice shares extensive synteny among other cereals thereby increasing the utility of this system (Devos and Gale, 2000). These, together with availability of ~28,000 full length cDNAs, a large number of expressed sequence tags, yeast artificial chromosomes, bacterial artificial chromosomes, P1-derived artificial chromosomes, libraries and rich forward and reverse genetics resources (Hirochika *et al.*,2004) have made rice a worthy forerunner among the plants especially among the cereals.

In our studies, two rice varieties were used: Khao Dawk Mali (KDML) 105 and FL530 which are the southeast Asian *Indica* rice cultivars. KDML105, commonly known as "Jasmine rice" or "Thai Hom Mali rice", is a photoperiod-sensitive cultivar and is well adapted to the rainfed environments in which the fields are normally low fertility, frequently experiencing drought and salt stresses and also often attacked by diseases and insect pests. FL530 is a salt tolerant rice variety that was developed by the Rice Gene Discovery Unit, in collaboration with the National Center for Genetic Engineering and Biotechnology (BIOTEC) and the DNA Technology Laboratory (DNATEC) of Kasetsart University Kamphaeng Sean Campus. To obtain FL530, the hybrid strain between pokkali

and IR29 was crossed with KDML105 and the resulting progeny was backcrossed with KDML105 for three more generations. The salt tolerant phenotype is believed to derive from the first chromosome of pokkali. Therefore, FL530 contains the genetic background similar to KDML105, but it has higher salt tolerance, which is suitable for comparative studies.

Ca^{2+} serves as an intracellular messenger in many cellular processes including plant responses to environmental stresses such as salinity, drought and cold. These stresses have been shown to induce transient elevation of the cytoplasmic Ca^{2+} concentration level. Elevation of the Ca^{2+} concentration is detected by calcium sensor proteins. The classical calcium sensor is calmodulin (CaM), which regulates activity of its protein targets in a calcium-dependent manner. In plants, CaM may play an important role in transducing Ca^{2+}-mediated signal from salt stress into appropriate adaptive cellular responses. In this research, the family of Ca^{2+}-binding proteins especially CaM and its related Ca^{2+}-sensors were identified and analyzed from the rice genome by database searching and bioinformatic tools. And then, the expression pattern of these genes was studied in rice plants grown under salt stress to determine which genes are important to response to salt stress. In addition, salt stress increases the expression level of abscisic acid (ABA) biosynthetic genes mediated by a Ca^{2+} signal. In turn, ABA acts as a messenger for salt stress, also likely through a Ca^{2+} signal. Therefore, the expression patterns of *OsCam1-1* were examined in salt sensitive and salt tolerant rice, KDML105 and FL530 respectively, under salt stress and with exogenous ABA application. The objectives of this study are:

1. To search and analyze the *calmodulin* (*Cam*) gene cluster from databases of the rice genome.
2. To study *calmodulin* gene expression in KDML105 and FL530, a KDML105-derived salt-tolerant line cultured in NaCl-containing media.