Chapter: 2

REVIEW OF LITERATURE
Water is a fundamentally important component of the metabolism of all living organisms. As a universal solvent it facilitates many vital biological reactions in the living system. Salinization is the accumulation of water-soluble salts in the soil solum (the upper part of a soil profile) or regolith (the layer or mantle of fragmental and unconsolidated rock material) to a level that would affect agricultural production. Salinity affects almost every aspect of the physiology and biochemistry of plants and significantly reduces yield. High exogenous salt concentrations affect seed germination, water deficit, cause ion imbalance of the cellular ions resulting in ion toxicity and osmotic stress (Khan et al., 2002; Khan and Panda, 2008). Plants undergo continuous exposure to various biotic and abiotic stresses in their natural environment. To survive under such conditions, plants have evolved intricate mechanisms to perceive external signals, allowing optimal response to environmental conditions. Phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene
(ET), and abscisic acid (ABA) are endogenous, low-molecular-weight molecules that primarily regulate the protective responses of plants against both biotic and abiotic stresses via synergistic and antagonistic actions.

The most important process that is affected in plants, growing under saline conditions, is photosynthesis. Reduced photosynthesis under salinity is not only attributed to stomata closure leading to a reduction of intercellular CO$_2$ concentration, but also to non-stomata factors. There is strong evidence that salt affects photosynthetic enzymes, chlorophylls and carotenoids (Stepien and Klobus, 2006). Salinity reduces the ability of plants to utilize water and causes a reduction in growth rate, as well as changes in plant metabolic processes (Munns, 2002). The complexity of the plant responses to salt stress can be partially explained by the fact that salinity imposes both hyperionic and hyperosmotic stress on the plants (Glenn et al., 1999) and inhibits plant growth by inhibiting cell division and expansion, imbalance of the cellular ions, change of cell volume or turgor pressure and activity and stability of macromolecules. The degree of growth inhibition due to osmotic stress depends on the severity of the treatment, time scale of the response and tissue and species in question. Whereas mild osmotic stress is known to cause inhibition of growth of leaves and stems, it does not affect the growth of roots (Westgate and Boyer, 1985; Sharp et al., 1988; Spollen et al., 1993). Osorio et al. (1998) have suggested that the inhibition of shoot growth during water deficit could contribute to solute accumulation and thus eventually to osmotic adjustment. On the other hand, continuation of root growth under drought stress has been suggested to be an adaptive mechanism that facilitates water uptake from deeper soil layers (Munns et
It has been suggested that continued root growth may provide additional surfaces for sequestration of toxic ions, leading to lower salt concentration.

An initial rapid and transient drop in growth rate followed by a gradual recovery to a new reduced rate of growth upon exposure to salinity in plant leaves has been recorded in maize (Cramer and Bowman, 1991; Neumann, 1993), rice (Yeo et al., 1991), wheat (Passioura and Munns, 2000) and barley (Fricke et al., 2006). Yeo et al. (1991) have ascribed the initial responses of plants to salt stress to change in cell water relations imposed by external osmotic pressure. Plants sense salt stress through both ionic (Na\(^+\)) and osmotic stress signals. Zhu (2003) has suggested that plants sense excess Na\(^+\) levels either on the surface of the plasma membrane protein or within the cell by membrane proteins including Na\(^+\) sensitive enzymes. It has been suggested that in addition to its role as an antiporter, the plasma membrane Na\(^+\)/H\(^+\) antiporter SOS1, having 10 to 12 transmembrane domains and a long cytoplasmic trail, may also act as a Na\(^+\) sensor (Zhu, 2003). Sanders et al. (1999) have observed that entry of Na\(^+\) through nonspecific ion channels under salinity caused membrane depolarization that activates Ca\(^{2+}\) channels generating Ca\(^{2+}\) oscillations. They have suggested that the Ca\(^{2+}\) oscillations could act as stress signals for the plant during salt stress. Addition of external Ca\(^{2+}\), however, alleviated the salt-induced reduction of growth in the root of maize and sorghum (Cramer and Läuchli, 1986; Cramer et al., 1988; Colmer et al., 1996).

Given the multiplicity of stress signals, many different sensors are expected, although none have been confirmed for cold, drought, or salinity. In plants many of the different types of stresses viz. cold, drought, and salt stimulate the accumulation
of compatible osmolytes and antioxidants (Hasegawa et al., 2000). Knight and Knight (2001) have suggested that the multiple signal pathways associated with different types of stress can operate independent of each other and may even modulate other pathways. Each of these pathways is expected to have a component of input signal and a component of output response. For the ionic aspect of salt stress, the input of the SOS pathway has been suggested to be the likely excess intracellular or extracellular Na⁺ (Zhu, 2000). The output responses are expression and activity changes of transporters for ions such as Na⁺, K⁺, and H⁺. Zhu (2001) has suggested that the input for osmotic stress signaling pathway could be change in turgor with a corresponding output response being activation of osmolyte biosynthesis enzymes as well as osmolyte transport systems. Xiong et al. (2002) divided the signal transduction pathways in plants under environmental stresses into three major types: (i) Ca⁴⁺-dependent salt overly sensitive (SOS) signaling that results in ion homeostasis (ii) Ca⁴⁺-dependent signaling that leads to activation of LEA-type genes such as DRE/CRT class of genes and (iii) osmotic/oxidative stress signaling that makes use of MAPK modules.

Plants respond to salt stress induced disturbances in ionic homeostasis by restricting salt intake, increased electrolyte leakage and maintenance of a favorable K⁺/Na⁺ ratio in the cytosol (Niu et al., 1995; Serrano et al., 1999). Salt stress is known to increase the levels of free cytosolic Ca⁴⁺ either through influx from the apoplastic space or as a consequence of release from the Calcium sequestration compartments (Knight, 2000; Sanders et al., 1999). Schroeder et al. (2001) have indicated that inositol polyphosphates, cyclic ADP ribose and NADP could act as
second messengers in the salt stress signaling cascade and induce release of Ca\(^{2+}\) from sequestered compartments in cells. Elevated levels of free cytosolic calcium have been shown to reduce binding of Na\(^{+}\) ions to cell walls and plasma membranes, relieve membrane leakiness and prevent salt-induced decline in cell elongation (Stassart et al., 1981; Cramer et al., 1985; Kurth et al., 1986; Lynch et al., 1987; Zidan et al., 1990; Picchioni et al., 1991). However, Bliss et al. (1986) have suggested that supplemental Ca\(^{2+}\) alleviated deleterious effects of salt by mitigating the toxic effects of Na\(^{+}\) ions rather than the osmotic effects associated with salt stress. Ca\(^{2+}\) has been reported to improve germination and plumule emergence (Bliss et al., 1986), root elongation (Cramer et al., 1986; Nakamura et al., 1990; Zidan et al., 1990), shoot growth (Cramer et al., 1989; Grieve and Fujiyama, 1987; Grieve and Maas, 1988; Maas and Grieve, 1987; Subbarao et al., 1990; Yeo et al., 1991), prevent nuclear deformation and degradation (Katsuhara and Kawasaki, 1996), increase uptake and transport of K\(^{+}\) (Cramer et al., 1985, 1987; Grieve and Fujiyama, 1987; Nakamura et al., 1990; Subbarao et al., 1990) and reduce Na\(^{+}\) accumulation (Cramer et al., 1987, 1989; Ehret et al., 1990; Grieve and Fujiyama, 1987; Grieve and Maas, 1988; Maas and Grieve, 1987; Subbarao et al., 1990; Zidan et al., 1991) in plants during salt stress.

While Liu and Zhu (1998) and Chinnusamy et al. (2005) have attributed the effect of Ca\(^{2+}\) on Na\(^{+}\) and K\(^{+}\) transport SOS signaling pathway, Shi et al. (2002) have shown that the sensor protein for this salt-induced calcium signature is the Ca\(^{2+}\)-binding protein SOS1 and SOS3. Loss of function of these proteins due to mutation
Fig. 2.1: Schematic representation of SOS pathway in cells.
has been shown to render the plants hypersensitive to salt stress (Wu et al., 1996; Shi et al., 2000; Shi et al., 2002). Halfter et al. (2000) have suggested that extracellular Ca$^{2+}$ could directly alter Na$^+$ influx into cells leading to activation of the SOS pathway via SOS3. Using Arabidopsis as a model system Liu et al. (2000) have proposed that salt stress signaling is perceived by the calcineurin-β-like Ca$^{2+}$ sensor SOS3. However, unlike the calcineurin-β in yeast that acts through activation of a protein phosphatase, SOS3 has been reported to interact with and activate protein kinase SOS2 (Halfter et al., 2000). Thus, SOS3 resembles an adapter or scaffold protein that mediates the interaction of SOS2 with other proteins such as ion transporters. While the increased levels of cytosolic calcium are known to be perceived by various calcium-binding proteins viz. CDPKs and the SOS3 family of Ca$^{2+}$ sensors, Knight et al. (1997) have suggested that CDPK were the prime candidates that linked calcium signal to downstream responses of osmotic stress. Osmotic stress-induced CDPKs have been reported from several plants including rice (Saijo et al., 2000; Kawasaki et al., 2001; Oztur et al., 2002), Arabidopsis (Sheen, 1996), alfalfa (Munnik et al., 1999; Kiegerl et al., 2000) and tobacco (Elizabeth and Zhang, 2000; Yang et al., 2001; Zhang and Klessig, 2001). Besides CDPKs MAPKs are also known to be components of intracellular signal modules that mediate signal transduction from the cell surface to the nucleus. There is accumulating evidence indicating that plants rapidly activate MAPK when exposed to multiple abiotic stress stimuli (Ligterink and Hirt, 2001; Kiegerl et al., 2000). MAPKs are known to be activated in response to drought and other environmental stresses (Zhu, 2002; Agrawal et al., 2003). Xiong and Yang (2003) have shown that
the activated MAPKs are transported into the nucleus, where they phosphorylate and activate specific downstream signaling components, such as transcription factors to induce altered gene expression. Osmotic stress signaling MAPK modules which have been identified from different systems include SIMK and SIMKK–SIMK55 from alfalfa and Nt MEKZ–SIPK/ WIPK and SIPK from Nicotiana tobacum, NPK1 from Arabidopsis thaliana (Munnik et al., 1999, Kiegerl et al., 2000; Kovtun et al., 2000; Mikolajczyk et al., 2000; Liu et al., 2000; Yang et al., 2001; Zhang and Klessig, 2001).

In addition to serving important role as components of plasma membranes, membrane phospholipids constitute a dynamic system that generate a multitude of signal responses during stress (Munnik and Meijer, 2001). Like ROS, low levels of phospholipid messengers such as IP₃, DAG, PA are known to activate downstream adaptive responses (Sang et al., 2001). IP₃ has been shown to induce release of Ca²⁺ from isolated vacuoles or tonoplast vesicles thereby increasing the levels of free cytosolic calcium in cells (Schumaker and Sze, 1987; Munnik et al., 1998 and DeWald et al., 2001). IP₃ has also been shown to induce increase in the levels of free cytosolic calcium in guard cells and consequent stomatal closure (Sanders et al., 1999). Wu et al. (1997) have suggested that increased level of cytosolic calcium cause by IP₃ could be one of the components in the signaling cascade which caused expression of osmotic stress-responsive genes. Like IP₃, other inositol phosphates such as IP₆ and I(1,3,4)P₃ have also been reported to be involved in release of Ca²⁺ from internal stores (Lee et al., 1996; Lemtiri-Chlieh et al., 2000).

Several studies have reported that inositol 4,5-bisphosphate is the primary and
immediate catabolite of $^3$H-labeled IP$_3$ in plants (Joseph et al., 1989; Drobak et al., 1991; Brearley et al., 1997), suggesting that in these plants, IP$_3$ was first hydrolyzed through a 1-phosphatase pathway. However, the Ins1Pase responsible for this early termination of the IP$_3$ signal in plants has not been identified. While the activity of FRY1/SAL1 in the hydrolysis of Ins(1,4)P$_2$ and inositol 1,3,4-trisphosphate [Ins(1,3,4)P$_3$] had been demonstrated earlier in Arabidopsis thaliana (Quintero et al., 1996), its ability to hydrolyze IP$_3$ was not known. Using IP$_3$ as a substrate, FRY1 recombinant protein was found to have a measurable albeit limited activity [approx. 13% relative to its ability to hydrolyze Ins(1,4)P$_2$ or Ins(1,3,4)P$_3$] (Xiong et al., 2001c). The in vivo activity of FRY1 on IP$_3$ and its significance in overall IP$_3$ metabolism have yet to be determined. Measurement of IP$_3$ levels in fry1 and wild-type plants treated with ABA indicated that, whereas ABA induced a transient increase in IP$_3$ levels in wild-type plants, the IP$_3$ levels in fry1 mutant plants were higher and more sustained (Xiong et al., 2001c). Sustained IP$_3$ levels likely contributed to the enhanced expression of stress-responsive genes in fry1 mutant plants.

Accumulating evidence suggests that phospholipase D (PLD) is also involved in the transduction of stress signals. PLD hydrolyzes phospholipids to generate phosphatidic acid (PA), another second messenger in animal cells that can activate PI-PLC and protein kinase C (English, 1996). Wang (1999) has suggested that PA may also serve as a messenger in plants. Jacob et al. (1999) have demonstrated PLD activity mediated ABA-induced stomatal closure in guard cell protoplasts. Drought and hyperosmolarity have been shown to activate PLD and lead to transient increases
in PA levels in plants (Frank et al., 2000; Munnik et al., 2000; Katagiri et al., 2001). It has been suggested that PLD activation might be taking place through a G-protein (Frank et al., 2000) and the activation may be independent of ABA (Frank et al., 2000; Katagiri et al., 2001).

Xiong et al. (2001) and Burnette et al. (2001) have demonstrated the role of ABA in increasing IP3 levels in *Vicia faba* guard cell protoplasts and *Arabidopsis* seedlings indicating thereby the involvement of ABA as a component in the stress response signaling cascade. Sanchez and Chua (2001) and Burnette et al. (2001) have demonstrated that inositol-5-phosphatase could regulate ABA signal transduction pathway through changes in levels of endogenous IP3. Takahashi et al. (2001) have shown that suppression of increase in levels of IP3 because of inhibition of PI-PLC activity inhibited the osmotic stress induction of the stress-responsive genes *RD29A* and *COR47*.

Xiong et al. (2001) have shown that mutations in the *FRY1* gene encoding an inositol polyphosphate-1-phosphatase resulted in enhanced ABA induced gene transcription in *Arabidopsis thaliana*. Despite increased IP3 levels and enhanced expression of *fry1* gene, the mutants were more susceptible to damage by salt, drought, or freezing stress. Xiong et al. (2001) have postulated that the enhanced expression was likely a compensatory mechanism to limit or repair stress injury. In this regard, FRY1 gene product might represent an interesting point of crosstalk between the stress homeostasis and detoxification pathways. Hyperosmotic stress has also been reported to stimulate PLA2 activity thereby generating lyso-phospholipids and free fatty acids in algae (Einspahr et al., 1988; Meijer et al., 1999). Munnik and
Fig. 2.2: Schematic representation of phospholipid signaling pathway in cells during salt stress.
Meijer (2001) have suggested that this novel lipid messenger may have a role in osmoregulation by stimulating tonoplast H^+-ATPase activity in the cells under stress.

ABA plays a crucial role in higher plants in their response to various environmental stresses and serves as a regulatory link between stress factors and plant responses (Seemann and Sharkey, 1987; Chandler and Robertson, 1994). Physiological studies and molecular analysis have demonstrated that ABA may regulate the adaptation of plants to environmental stresses (Stewart and Voetberg, 1985; Skriver and Mundy, 1990). The increase in endogenous ABA levels under water stress or application of exogenous ABA is often accompanied by enhanced synthesis of dehydration-related proteins and other compatible solutes such as proline, glycine-betaine, cyclitols and enhanced expression of a number of specific genes in plant tissues (Hanson et al., 1985; Stewart and Voetberg, 1985; Gomez et al., 1988; Mundy and Chua, 1988; Bray, 1988; Blackman et al., 1991, 1992; Parcy et al., 1994; Seo et al., 1995; Beardmore and Charest, 1995; Liotenberg et al., 1999; Sun et al., 1999; Xiong et al., 2001). ABA is also known to play an important role in acquisition of desiccation tolerance in seeds during embryo maturation (Quatrano, 1987; Blackman et al., 1991, 1992; Meurs et al., 1992). Exogenously applied ABA has been shown to be able to induce desiccation tolerance in immature zygotic embryos (Wakui et al., 1994; Blackman et al., 1992) as well as somatic embryos (Park et al., 1988; Bochicchio et al., 1991; Etienne et al., 1993). Even though many of the osmotic stress responsive genes are known to be induced by ABA (Liotenberg et al., 1999), AA03 (Seo et al., 1995), and ABA3 (Xiong et al., 2001). Shinozaki and
Yamaguchi-Shinozaki (1997) have showed that induction of many other osmotic stress responsive genes could be independent ABA accumulation in the cells. There is evidence that although ABA does not activate the DRE in the RD29A promoter, it may be required for full activation of DRE by osmotic stress (Yamaguchi-Shinozaki and Shinozaki, 1994). Xiong et al. 2001 have proposed that activation of DRE by DREB2A and related transcription factors may require ABA-dependent factor(s). An analysis of double mutants between fry1 and abal or abil indicated that the cold or osmotic stress hypersensitivity in the mutant is not dependent on ABA (Zhu et al., 2002). Sharing of ABA-dependent and ABA-independent pathways may also occur downstream of the first stress recognition and signaling events, and/or a gene may contain both DRE and ABRE elements in its promoter. Several genes that are upregulated under drought conditions are known to contain a conserved ABRE in their promoter region (Qin and Zeevart, 1999; Uno et al., 2000).

A secondary effect of salt stress is the increase in levels of reactive oxygen species (ROS) (Smirnoff, 1998; Bartels, 2001; Apel and Hirt, 2004). ROS’s are partially reduced forms of atmospheric oxygen, which are produced in vital processes such as photorespiration, photosynthesis and respiration (Mittler, 2002; Uchida et al., 2002). To produce water in these processes, four electrons are required for perfect reduction of oxygen. But ROS typically results from the transference of one, two and three electrons, respectively, to O2 to form superoxide (O2^{-}), peroxide hydrogen (H2O2) and hydroxyl radical (HO') (Mittler, 2002). These species of oxygen are highly cytotoxic and can seriously react with vital biomolecules such as lipids, proteins, nucleic acid, etc, causing lipid peroxidation, protein denaturing and DNA
mutation, respectively (Breusegem et al., 2001; Scandalios, 1993; Quiles and Lopez, 2004). Increased levels of ROS cause oxidative stress in the tissues and consequent damage to proteins, lipids and nucleic acids; the magnitude of damage depending upon the balance between the formation of ROS and their removal by the antioxidative scavenging systems (Hernandez and Almansa, 2002). Even though ROS have been implicated in the regulation of several physiological processes such as cell proliferation (Shibanuma et al., 1990), differentiation (Allen and Balin, 1989), senescence (de Haan et al., 1996), and apoptosis (Mignotte and Vayssiere, 1998), activation of defense responses (Dat et al., 2000; Mittler, 2002) at low concentrations, they are highly cytotoxic at higher concentrations and induce DNA damage, lipid peroxidation, and protein degradation (Sun, 1990; Scandalios, 1993; Breusegem et al., 2001; Quiles and Lopez, 2004). The capacity to tolerate abiotic stress is also known to be associated with a more efficient antioxidative system (Foyer et al., 1994; Gosset et al., 1996; Hernandez et al. 1993, 1995, 1999, 2002; Bor et al., 2003; Vaidyanathan et al., 2003). The enzymatic and nonenzymatic systems involved in maintenance of ROS balance in plants include enzymes like ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) as well as low molecular mass antioxidants viz. glutathione and ascorbate (Foyer et al., 1994). Other enzymes that are important in the ROS scavenging system and function in the ascorbate-glutathione cycle are glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Candan and Tarhan, 2003; Yoshimura et al., 2000). Aziz and Larher (1998) have observed a sharp increase in the activities of superoxide dismutase and ascorbate peroxidase in
osmotically stressed leaf discs of rape plants. Increases in the activities of ascorbate peroxidase, superoxide dismutase and glutathione reductase have also been observed in the drought tolerant wheat cultivar 'Massai' when the leaves were subjected to osmotic stress in vitro (Lascano et al., 2001). Alscher et al. (2002) have suggested that since the antioxidant systems play an important role in scavenging ROS, their improvement could increase the ability of plants to tolerate adverse environmental conditions. Lai et al. (2007) have demonstrated a marked increase in the activities of SOD, CAT, APX, GPX and DHAR in leaf discs treated with polyethylene glycol (PEG). The increase in activities of the enzymes in leaf discs of plants transformed with the clone PSAG12-IPT was much higher than that in the untransformed controls particularly at 40% PEG treatment. Higher activities of SOD, CAT, APX, GPX and DHAR in leaf discs of plants transformed with the clone PSAG12-IPT treated with PEG coincided with decrease in the TBARS concentration, suggesting that oxidative damage induced by osmotic stress could be alleviated by transformation by the chimeric gene PSAG12-IPT. This result was consistent with the observation of Dertinger et al. (2003) who have reported that the activities of antioxidative enzymes (APX and GR) in old leaves of PSAG12-IPT modified tobacco plants were higher than that in the wild-type leaves.

Amongst the reactive oxygen species which mediate responses to various stimuli, H$_2$O$_2$ seems best suited to play the role of signaling molecule due to its higher stability and longer half-life. Yu et al. (2002, 2003) suggest that H$_2$O$_2$ is a signal mediator for programmed cell death of plants as a response to pathogens, elicitors, and hormones (Desikan et al., 1998; Mittler et al., 1999; Solomon et al.,
Fig. 2.3: Schematic representation of antioxidative pathways in cells during salt stress.
Furthermore, a number of studies indicate that H$_2$O$_2$ is synthesized in response to application of exogenous ABA and that H$_2$O$_2$ mediates ABA induced stomatal closure in leaves (Guan et al., 2000; Pei et al., 2000; Desikan et al., 2001a). H$_2$O$_2$ has also been reported to be involved in regulating differential expression of genes during stress including genes encoding potential transcription factors. These results suggest that transcription factors mediate further downstream H$_2$O$_2$ responses and finally induce physiological changes to promote adaptation to stresses. Yang and Poovaiah (2002) have demonstrated that treatment with H$_2$O$_2$ activated Ca$^{2+}$ channels leading to elevation of cytCa$^{2+}$ level. Increased levels of cytosolic calcium would activate the calcium sensor ‘calmodulin’ which would through a downstream signaling cascade induce the expression of gene coding for catalase for scavenging the H$_2$O$_2$. Evidence suggests that membranes are the primary sites of salinity injury to cells and organelles (Candan and Tarhan, 2003). ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalemma or intracellular organelles (Karabal et al., 2003; Stewart and Bewley, 1980).

Current evidence supports the concept that ROS represent a significant point of convergence between pathways that respond to both biotic as well as abiotic stresses. Nevertheless, our current understanding of ROS participation in crosstalk between these pathways is very limited. Moreover, the view that the ABA mediated abiotic stress signaling potentially takes precedence over biotic stress signaling (Ghassemian et al., 2000) also supports the notion that water stress more significantly threatens plant survival than pathogen infection. To date, the biological
significance of crosstalk between signaling pathways that operate under stress conditions and the mechanisms that underlie this crosstalk remain obscure. Thus, dissecting the genetic network that regulates ROS signaling in response to such stressful conditions merits extensive future study. When combined, the results of large-scale transcriptome, proteome, and metabolome analyses in plants could enable the elucidation of the ROS network components that govern multiple stress signaling pathways.