9 Plant salt tolerance

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Abstract

Soil salinity adversely affects crop productivity and quality. The success of breeding programs aimed at salinity tolerant crop varieties is limited by the lack of a clear understanding of the molecular basis of salt tolerance. Recent advances in genetic analysis of *Arabidopsis* mutants defective in salt tolerance, and molecular cloning of these loci, have showed some insight into salt stress signaling and plant salt tolerance. Salt stress-induced cytosolic calcium signals are perceived by SOS3, which is a calcium sensor protein. SOS3 is constitutively myristoylated and associated with the plasma membrane. The SOS3 activates SOS2, a ser/thr protein kinase, in a calcium dependent manner. The active SOS3-SOS2 kinase complex activates SOS1, a Na^+/H^+ antiporter on the plasma membrane and also upregulates *SOS1* gene expression; this results in Na^+ efflux and ion homeostasis. Transgenic analysis showed a tonoplast-located Na^+/H^+ antiporter mediates sodium sequestration into the vacuole, and this forms an important part of the salt tolerance mechanism. Evidence also implicates a putative osmosensory histidine kinase (AtHK1)-MAPK cascade and its negative regulators (AtMKP1) in salt stress signaling that probably leads to osmotic homeostasis and ROS scavenging. ABA-mediated regulation of stress proteins and plant growth are also important for plant salt tolerance, but the signaling pathway is poorly understood.

9.1 Introduction

Soil salinity predates human civilization and is probably a cause of the breakdown of the ancient Sumerian civilization (Jacobson and Adams 1958). Today salinity remains a major abiotic stress that adversely affects crop productivity and quality (Boyer 1982). Saline soil is characterized by toxic levels of chlorides and sulfates of sodium. The electrical conductivity of saturation extracts of saline soil is more than 4.0 dS/m (≈ 40mM NaCl; Marschner 1995). The problem of soil salinity is increasing owing to 1) the use of poor quality water for irrigation, 2) improper drainage in canal-irrigated wetland agro-ecosystems, 3) entry of seawater during cyclones in coastal areas, and 4) salt accumulation in the root zone in arid and semi-arid regions due to high evaporative demand and insufficient leaching of ions as the rainfall is inadequate.

Sodium is an essential micronutrient for some of the C_4 photosynthetic plants, which import pyruvate into mesophyll chloroplasts by a Na^+/pyruvate co-

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transporter (Ohnishi et al. 1990). However, most crop plants are natrophobic. Salinity is detrimental to plant growth as it causes 1) nutritional constraints by decreasing uptake of phosphorus, potassium, nitrate and calcium, 2) ion cytotoxicity mainly due to Na^+, Cl^- plus SO_4^-, and 3) osmotic stress (reviewed by Zhu 2001, 2002). Na^+ competes with K^+ in biochemical reactions, which is inimical to cellular processes. Under salinity, ions like Na^+ and Cl^- penetrate the hydration shells of proteins and interfere with the non-covalent interactions between amino acids of proteins. This leads to conformational changes and loss of function of proteins. Ionic toxicity, osmotic stress, and nutritional defects under salinity may lead to metabolic imbalances, which result in oxidative stress (Zhu 2001). Plant salt tolerance mechanisms can be grouped into 1) cellular homeostasis which includes ion homeostasis and osmotic adjustment, 2) stress damage control and repair, or detoxification, and 3) growth regulation (Zhu, 2001). Considerable efforts have been made to unravel plant salt tolerance mechanisms with the ultimate goal of improving the crop productivity in saline soils. Here we discuss molecular and genetic evidence concerning the perception of salinity stress by plants, cellular signal transduction, and effectors of salt stress tolerance.

9.2 Sodium entry into plant cells

The membrane potential difference at the plasma membrane of plant cells is -140 mV, which favors passive transport of Na^+ into cells, especially with high extracellular Na^+ concentrations. Excess extracellular Na^+ enters the cell through both the transporter HKT1 and non-selective cation channels/transporters, which results in a decrease in the K^+/Na^+ ratio in the cytosol. The wheat high affinity K^+ transporter HKT1 appears to act as a low affinity Na^+ transporter (Rubio et al. 1995; Gorham et al. 1997). Expression of the *Arabidopsis* homolog of wheat *HKT1* (*ATHKT1*) in *Xenopus* oocytes mediated Na^+ influx, which suggested that ATHKT1 might be involved in Na^+ influx in plants (Uozumi et al. 2000). Eucalyptus *EcHKT1* and *EcHKT2* when expressed in oocytes showed both Na^+ and K^+ uptake, but the permeability to Na^+ was greater than that for K^+ when the extracellular concentration of Na^+ and K^+ were equal (Liu et al. 2001). These results suggest that in plants in general, HKT1 might be involved in low affinity Na^+ influx. In rice, the contribution of carrier protein-mediated Na^+ uptake is less significant than the apoplastic pathway under high salinity conditions (Yadav et al. 1996; Garcia et al. 1997). Quantitative trait loci (QTL) and inheritance analysis in rice revealed that genes that control Na^+ uptake are different from that of K^+ uptake (Garcia et al. 1997; Koyama et al. 2001). Silica deposition in the endodermis and rhizodermis, and the polymerization of silicate via colloidal silica to silica gel or polysilicic acid throughout the root apoplast, appears to block Na^+ uptake through the apoplastic pathway in the roots of rice (Yeo et al. 1999). Hence, the entry of Na^+ in rice under salinity is expected to be regulated significantly by genes that affect root development and silicon uptake. However, in wheat, sodium/potassium selectivity by carrier proteins in the root appears to be a major determinant of salt

tolerance (Gorham et al. 1997). Thus, the entry of Na^+ into plant root cells can be affected by the regulation of K^+/Na^+ transporter HKT1 and non-selective cation channels, and by regulation of genes involved in root development and silicon polymerization-mediated blockage of the apoplastic route. Plant species-specific differences and the regulatory mechanism of Na^+ entry into plant roots under salinity need to be understood.

9.3 Input signals of salt stress

Salt stress affects cellular ion homeostasis as well as osmotic homeostasis. Excess Na^+ and Cl^- ions may lead to conformational changes in protein structure and/or changes in the plasma membrane electrical potential, while osmotic stress leads to turgor loss and cell volume change. Hence, excess ions (Na^+ and Cl^-) and osmotic stress-induced turgor change may act as inputs for salt stress signaling. The candidate sensors of ionic stress may include ion channels/transporters and ion binding proteins on the plasma membrane or at intracellular locations (Zhu 2002). Under High Na^+ concentrations, Na^+ may enter cells through non-specific ion channels, which might cause membrane depolarization. A change in membrane polarization could also signal salt stress, as it is known to activate Ca^{2+} channels (Sanders et al. 1999). Loss of turgor leads to a cell volume change and retraction of the plasma membrane from the cell wall. As the membrane retracts from the cell wall, membrane bound receptor kinases, ion transporters/channels, transmembrane proteins that are in contact with cell wall, and integrin-like proteins may undergo conformational changes or cluster together, and hence these proteins may act as sensors of osmotic stress. Integrins and the F-actin cytoskeleton have been implicated in the sensing of cell volume changes in mammalian cells. Regulation of microtubule organization by turgor pressure was shown in *Spirogyra* sp. (Iwata et al. 2001). Microtubules and microfilaments of the cytoskeleton have been implicated in signaling under cold stress in plants (reviewed by Viswanathan and Zhu 2002), and the pattern of microtubular organization under cold stress differs from that of ABA (Wang and Nick 2001). Since the cytoskeleton connects different organelles of the cell with the plasma membrane, it can sense cell volume change under osmotic stress and transduce it to internal Ca^{2+} channels or other signaling components. Salinity induces the biosynthesis and accumulation of the plant stress hormone abscisic acid (ABA; Jia et al. 2002) and also induces accumulation of reactive oxygen species (ROS; Smirnoff 1993; Gomez et al. 1999; Hernandez et al. 2001). Current evidence suggests that the primary salt stress signals (ionic and osmotic stress) are transduced through Ca^{2+} as well as receptor kinase pathways, while the secondary salt stress signals such as ABA and H_2O_2 also regulate plant salt tolerance.

9.3.1 Calcium signalling

Cytosolic Ca^{2+} oscillations, generated from extracellular and/or intracellular Ca^{2+} stores, act as a second messenger in cold, drought, and salt stresses (Sanders et al. 1999; Knight 2000). Calcium oscillations in plant cells vary, depending upon the type of stress (Kiegle et al. 2000), rate of stress development (Plieth et al. 1999), previous experience of stresses/cycles (Knight et al. 1997), and tissue type (Kiegle et al. 2000). Cytosolic Ca^{2+} oscillations occur within 5-10 seconds of salt stress, persist up to 1 to 10 minutes and, hence, are thought to be one of the earliest events in salt signaling (Lynch et al. 1989; Knight et al. 1997). Therefore, it is essential to analyze how such Ca^{2+} signatures are generated by a salt stress signal, and what are the components downstream that decode salt stress-specific Ca^{2+} signatures.

Cytosolic Ca^{2+} signatures can be the net result of influx and efflux of Ca^{2+}. Calcium efflux occurs through Ca^{2+} ATPases and H^+/Ca^{2+} antiporters, while influx is controlled by Ca^{2+} permeable ion channels (Sanders et al. 1999). In animal cells, ligand-gated Ca^{2+} channels are regulated by inositol (1,4,5)-triphosphate (IP_3), cyclic adenosine 5'diphosphate ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate ($NAADP^+$). In plants, IP_3 and cADPR-gated Ca^{2+} channels are found in vacuolar and endoplasmic reticulum membranes (Allen et al. 1995; Wu et al. 1997), and NAADP-gated Ca^{2+} channels are found in the endoplasmic reticulum membrane (Navazio et al. 2000). In *Arabidopsis*, osmotic stress (NaCl or sorbitol) induces the synthesis of IP_3 to significantly higher levels within 1 minute of stress initiation, and it continues to increase for more than 30 minutes. Phospholipase C (PLC) hydrolyses phosphotidylinositol-4,5 bisphosphate into diacyl glycerol and IP_3, which are activators of protein kinase and calcium channels, respectively. Treatment with U-73122, an inhibitor of PLC, blocked IP_3 accumulation. The temporal pattern of IP_3 accumulation is similar to that observed for stress-induced calcium mobilization, implicating IP_3 in salt stress-induced Ca^{2+} signaling (DeWald et al. 2001; Takahashi et al. 2001). In cell cultures of *Arabidopsis*, a few seconds of osmotic stress (dehydration, mannitol or NaCl) caused a rapid and transient increase in IP_3 and expression of dehydration-inducible genes (*RD29A/LTI78/COR78* & *RD17/COR47*). This response was abolished when the cells were treated with inhibitors of PLC, such as neomycin and U73122, indicating the involvement of PLC and IP_3 in hyper-osmotic stress signaling (Takahashi et al. 2001).

Osmotic stress caused by NaCl/mannitol/sorbitol significantly increases cellular PtdIns(4,5)P_2 synthesis (Pical et al. 1999; DeWald et al. 2001), which is the substrate for cleavage by PLC to produce IP_3 (DeWald et al. 2001). Consistent with this, it has been shown that a *PLC* gene is also upregulated by osmotic stress (Hirayama et al. 1995). Salt stress-induced phosphatidyInositol (4,5)P_2 synthesis and cleavage into IP_3 may help in delayed Ca^{2+} signaling. Genetic evidence for the implication of IP_3 signaling in abiotic stresses including salinity came from the analysis of the *FRY1* locus of *Arabidopsis* (Table 1). *FRY1* encodes an inositol polyphosphate 1-phosphatase, which functions in the catabolism of IP_3. Upon ABA treatment, *fry1* mutant plants accumulated more IP_3 than did the wild type

Table 1. *Arabidopsis* mutants impaired in salt stress response

Mutant	Function	Salt tolerance	Reference
Salt overly sensitive 3 (*sos3*)	Ca^{2+} sensor	Hypersensitive	Liu and Zhu 1998
Salt overly sensitive 2 (*sos2*)	Protein kinase	Hypersensitive	Liu et al. 2000
Salt overly sensitive 1 (*sos1*)	Plasma membrane Na^+H^+ antiporter	Hypersensitive	Shi et al. 2000
Salt overly sensitive 4 (*sos4*)	pyridoxal kinase	Hypersensitive	Shi et al. 2002b
High affinity K transporter 1 (*hkt1*)	Plasma membrane Na^+ transporter	Suppresses salt sensitivity of sos3 mutant	Rus et al. 2001a
Fiery1 (*fry1*)	Inositol polyphosphate 1-phosphatase	Hypersensitive	Xiong et al. 2001b
Low expression of osmotically responsive genes 5 (*los5*)/ABA deficient 3 (*aba3*)	Molybdenum cofactor sulfurase	Hypersensitive	Xiong et al. 2001a
Low expression of osmotically responsive genes 6 (*los6*) /ABA deficient 1 (*aba1*)	zeaxanthin epoxidase	Tolerant during germination	Xiong et al. 2002a
SALOBREÑO (*sañ5*)/*abi4*	ABA insensitive 4	Tolerant during germination	Quesada et al. 2000
osm1	A protein similar to SNARE type mammalian syntaxins	Hypersensitive to salt and osmotic stress	Zhu et al. 2002
mkp1	MAPK phosphatase 1	Enhanced salt tolerance	Ulm et al. 2002
photoautotrophic salt tolerance1 (*pst1*)	Not yet cloned	Enhanced salt tolerance	Tsugane et al. 1999
t365	S-adenosyl-L-methionine phosphoethanolamine N-methyltransferase	Hypersensitive	Mou et al. 2002

plants. In wild type, IP_3 accumulation was transiently induced by ABA, while in *fry1* IP_3 accumulation was sustained, which suggest that IP_3 catabolism is mediated by FRY1. The *fry1* mutant is hypersensitive to ABA and salinity stress (Xiong et al. 2001b). The *Arabidopsis SAL1* gene, a homolog of *FRY1* conferred increased salt tolerance to yeast cells (Quintero et al. 1996). These results showed that IP_3 transient induced by salt and ABA is necessary for stress tolerance. In addition to IP_3-gated Ca^{2+} channels, stretch/mechanosensitive Ca^{2+} channels may also be involved in primary Ca^{2+} oscillations (Knight et al. 1997), as these Ca^{2+} channels can be activated immediately by a change in cell volume/turgor in salt stressed cells. Hence, salt stress-induced IP_3 oscillations are an integral part of

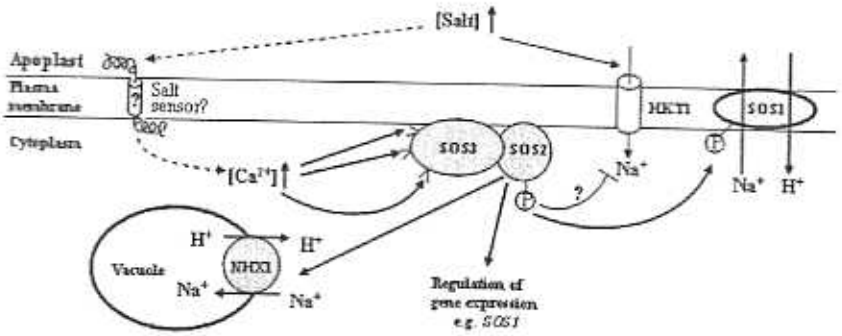

Fig. 1. The SOS pathway for ion homeostasis regulation under salt stress. Salt stress induced Ca^{2+} signals are perceived by SOS3, which activates the SOS2 kinase. Activated SOS2 kinase phosphorylates the SOS1 Na^+/H^+ antiporter, which then pumps Na^+ out of the cytosol. The SOS3-SOS2 kinase complex also regulates the transcript level of *SOS1* and other genes. The SOS3-SOS2 kinase complex may regulate Na^+ compartmentation by activating NHX1, and also may restrict Na^+ entry into the cytosol, e.g. by inhibiting the plasma membrane Na^+ transporter HKT1 activity.

Ca^{2+} signaling in salt stress. Engineered alterations in intracellular Ca^{2+} levels due to overexpression of the *Arabidopsis* vacuolar Ca^{2+}/H^+ antiporter gene (*CAX1*) in tobacco (Hirschi 1999), and the ionotrophic glutamate receptor (*GluR2*) in *Arabidopsis* (Kim et al. 2001) resulted in hypersensitivity to salt stress and other developmental abnormalities. This evidence strongly suggests that oscillations in intracellular Ca^{2+} levels form an integral part of plant salt tolerance.

9.3.2 Calcium sensors

Three major families of calcium binding proteins sense Ca^{2+} signals in plants (Liu and Zhu 1998; Harmon et al. 2000): 1) Calmodulins (CaM), which do not have enzymatic activity but transduce signals to CaM interacting proteins. Calmodulins contain four EF-hand domains responsible for Ca^{2+} binding, 2) Calcium dependent protein kinases (CDPKs), which contain CaM-like Ca^{2+}-binding domains and a kinase domain in a single protein, and 3) SOS3 and SOS3-like calcium-binding proteins (SCaBPs) (Liu and Zhu 1998; Guo et al. 2001). The specificity of Ca^{2+} signals may be achieved by the multiplicity of calcium sensors and their intracellular localization. The first genetic evidence for a calcium sensor protein mediated Ca^{2+} signaling in salt stress came from the analysis of the *salt overly sensitive 3* (*sos3*) mutant of *Arabidopsis* (Table 1, Fig. 1; Liu and Zhu 1998). The SOS3 mediated salt stress signaling in cellular ion homeostasis is discussed in the later part of this review.

The *Arabidopsis AtGSK1* gene, which encodes a protein similar to glycogen synthase kinase3, complemented yeast mutant DHT22-1a that is defective in both calcineurin (*SLN1* and *SHO1*) genes. Expression of *AtGSK1* in the yeast mutant

DHT22-1a also restored salt stress-induced expression of a Na^+-ATPase (*PMR2A*) gene. Moreover, *AtGSK1* is upregulated under ABA and salt stresses in *Arabidopsis*, suggesting the possible involvement of AtGSK1 in phosphoprotein-dependent salt stress signaling (Piao et al. 1999; Charrier et al. 2002).

The CDPK family of protein kinases contains a myristoylation and a calcium binding EF hand domain. The *Arabidopsis AtCDPK1* and *AtCDPK2* genes are induced by salinity and drought but not by low/high temperatures, suggesting that these two ATCDPKs might be involved in osmotic stress signaling (Urao et al. 1994). The involvement of CDPKs in stress-induced gene transcription was shown by Sheen (1996) in a maize leaf protoplast system transiently expressing barley *HVA1* promoter-driven synthetic green fluorescent protein. The barley *HVA1* gene, encoding a class 3 late embryogenesis-abundant protein, is induced by abiotic stresses such as drought, cold, heat, salinity, and ABA. Expression of *HVA1-SGFP* was significantly increased in the protoplasts incubated with Ca^{2+} and Ca^{2+} ionophore (ionomycin or A23187), but not by Ca^{2+} alone, indicating that Ca^{2+} entry is essential for *HVA1* expression. Further, maize protoplasts co-expressing the *HVA1-LUC* reporter and truncated *AtCDPK1* or *AtCDPK1a* (truncation of the regulatory domain of ATCDPKs results in Ca^{2+} independent, constitutive protein kinase activity) showed constitutive expression of *HVA1::LUC*. Mutated AtCDPK1, which lacks ATP binding activity, was unable to induce the *HVA1* promoter. These data indicate that protein kinase activity of AtCDPK1 is essential to activate the *HVA1* promoter. The Ca^+ requirement for induction of the abiotic stress responsive *HVA1* promoter in maize protoplasts suggests the involvement of AtCDPK1 in decoding Ca^{2+} signals under abiotic stresses in plants (Sheen 1996). Transgenic analysis showed that rice OsCDPK7 is a positive regulator of cold and salt/drought stress signaling. Rice transgenics overexpressing *OsCDPK7* under the control of the *CaMV 35S* promoter showed enhanced induction of a stress-responsive gene *RAB16A* in response to salinity/drought, and higher salt/drought stress tolerance, while transgenic lines in which OsCDPK7 was suppressed were hypersensitive to salt/drought stress (Saijo et al. 2000).

The *RAB16* (Skriver et al. 1991) and *HVA1* (Shen et al. 1996) genes have a G-box type ABRE *cis* element, which can be activated by bZIP transcription factors (Leung and Giraudat 1998). Overexpression of the catalytic domain of *ABI1 PP2C* inhibited the induction of *HVA1* transcription by ABA and ATCDPK1 (Fig. 2; Sheen 1996). These results suggest that the upregulation of *RAB16* and *HVA1* is mediated by CDPKs, probably through bZIP transcription factors, and is negatively regulated by the PP2C, ABI1. In addition to the activation of LEA-like genes, CDPKs also regulate transport proteins (aquaporins, ion channels and H^+-ATPase), which play pivotal roles in osmoregulation during osmotic and ionic stresses (Li et al. 1998; Lino et al. 1998). The Ca^{2+} requirement for activation of vacuolar chloride (VCL) and malate transporters in guard cells by CDPK was overcome by a constitutively active CDPK mutant (Pei et al. 1996).

In the common ice plant, CSP1 (a substrate protein for McCDPK1) was identified using yeast two-hybrid assays and wheat germ interaction assays. The phosphorylation of CSP1 *in vitro* by McCDPK1 required calcium. The deduced CSP1 amino acid sequence is similar to that of pseudo-response regulator-like proteins

that have a highly conserved DNA binding helix-loop-helix domain and a C-terminal activation domain. Salt stress induced co-localization of McCDPK1 and CSP1 in the nucleus of ice plants, but the targets of McCDPK1-CSP1 are not known (Patharkar and Cushman 2000). This study supports the possible involvement of CDPKs in salt stress signaling, which regulates ion homeostasis and gene expression.

9.3.3 Hybrid two-component receptor kinases

Two-component systems, consisting of a sensory histidine kinase and a response regulator, function as osmosensors in bacteria and yeast. The yeast Sln1, a transmembrane osmosensory histidine kinase, transfers the phosphoryl group to a His residue in an intermediary component Ypd1, and finally to an Asp residue in the response regulator, Ssk1, to inactivate it. High osmotic stress inhibits the autophosphorylation of Sln1 and hence the active non-phosphorylated form of Ssk1 accumulates, which in turn activates the Hog1 (high-osmolarity glycerol response 1) MAPK cascade. This leads to glycerol accumulation and osmoprotection as the Hog1 MAPK cascade positively regulates genes involved in glycerol biosynthesis. In addition to Sln1, another transmembrane osmosensor, Sho1, which is not a two-component system, is also known to regulate the Hog1 MAPK cascade under osmotic stress. Sln1 and Sho1 operate at different osmotic stress levels (Wurgler-Murphy and Saito 1997; Chang and Stewart 1998).

These studies gave the impetus to clone an osmosensory hybrid histidine kinase, *ATHK1*, from *Arabidopsis* (Urao et al. 1999). ATHK1 consists of both kinase and receiver domains in the same molecule, and complements the yeast mutant *sln1-ts* defective in osmosensing. Substitution in ATHK1 of the putative phosphorylation sites, either His within the kinase domain (His-508 to Val) or Asp within the receiver domain (Asp-1074 to Glu), caused it to fail to complement the yeast *sln1-ts* mutant. *ATHK1* confers high-osmolarity tolerance to the yeast double mutant (*sln1Δ sho1Δ*) lacking both osmosensors. This demonstrates that ATHK1 is active at low osmolarity, and is changed to the inactive form in response to high osmolarity, which activates the Hog1 MAPK pathway in yeast. Thus, ATHK1 has both structural and functional similarities to the yeast Sln1, suggesting that in plants ATHK1 acts as an osmosensor to transmit the stress signal to a downstream MAPK cascade. The transcript abundance of *ATHK1* is higher in roots than in other tissues under control conditions. *ATHK1* is upregulated under salt (250 mM NaCl) and low temperature (4°C) stresses. If the mechanism of osmosensing is a high osmotic pressure induced conformational change preventing autophosphorylation of ATHK1, newly synthesized ATHK1 under stress may be in the non-phosphorylated state leading to activation of a downstream signaling pathway, which is probably a MAPK cascade (Fig. 2; Urao et al. 1999).

In *Arabidopsis*, phosphorelay intermediates with His-containing phosphotransfer domains have been cloned (*ATHP1-3*). All three *ATHPs* can complement the yeast *ypd1* mutant, which implies that all ATHPs can transfer a phosphoryl group from SLN1 to SSK1 in yeast. Further, ATHP3 (=AHP1) transfers a phosphoryl

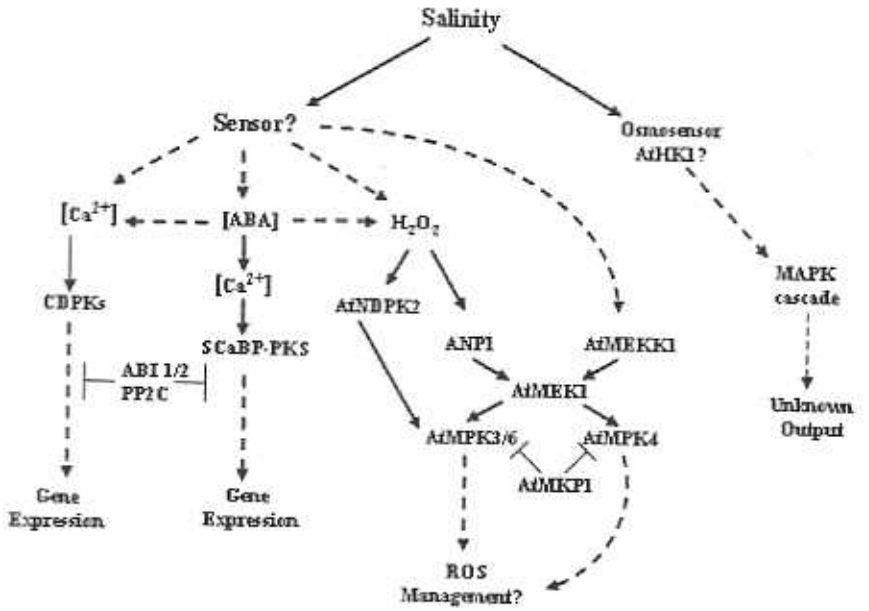

Fig. 2. Osmotic homeostasis and ROS detoxification under salt stress. Ca^{2+} signals sensed by CDPKs are transduced through unknown signaling intermediates, which induce genes encoding LEA-like proteins. ABA induced Ca^{2+} signals are perceived by SCaBPs, which activate PKS. The ABA signaling pathway upregulates osmolyte biosynthesis and genes encoding LEA-like proteins under salt stress. Ca^{2+} signaling through CDPKs and SCaBPs is under negative control of Protein Phosphatase 2C (ABI 1/2). High osmolarity may be perceived by AtHK1, which presumably transduces the signal through a MAPK pathway. Salt stress and reactive oxygen species (ROS) activated MAPK (ANP1 & AtMEKK1 = MAPKKK; AtMEK1=MAPKK; AtMPK3, 4 & 6 = MAPK) cascade may regulate oxidative stress management (Broken arrows indicate unknown signaling intermediates).

group from its His to the receiver domain of the putative two-component response regulators ARR3 and ARR4 *in vitro*. Thus, the phosphorelay intermediate, ATHP3, has the ability to accept a phosphoryl group from the osmosensor, ATHK1, and transfer its phosphoryl group to a response regulator, ARR4. Although functional complementation in yeast shows that *Arabidopsis* has ATHK1-ATHP3-ARR4 as a phosphorelay system (Miyata et al. 1998; Suzuki et al. 1998 & 2001; Urao et al. 1999), the MAPK cascade that transduces the osmotic stress signal and the target genes regulated by this putative hybrid two-component osmosensor system in higher plants are yet to be identified.

9.3.4 MAPK pathway

A canonical mitogen activated protein kinase (MAPK) module consists of a MAPK kinase kinase (MAPKKK), which activates a MAPK kinase (MAPKK) by

phosphorylation of Ser or Thr residues (Ser-X-X-X-Ser/Thr) within the catalytic core. Activated MAPKK activates MAPK by phosphorylation of both Thr and Tyr within the TXY consensus sequence in MAPK. Plant MAPKs are implicated in signaling development, cell division, hormones, biotic, and abiotic stresses. Salt stress quickly (within 5-10 minutes) activates MAPKs from alfalfa (*s*alt stress *i*nduced MAPK, SIMK; Munnik et al. 1999), tobacco (*s*alicylic acid *i*nduced MAPK, SIPK; Hoyos and Zhang 2000; Mikolajczyk et al. 2000), and Arabidopsis (ATMPK3, ATMPK4 and ATMPK6; Mizoguchi et al. 1996; Ichimura et al. 2000). The SIPK is also induced by salicylic acid and osmotic stress. The activation of SIPK is calcium and ABA independent (Hoyos and Zhang 2000). Alfalfa SIMK-interacting SIMKK (=MAPKK) was identified using the yeast two hybrid screen. SIMKK activated SIMK and the activation was enhanced by salinity (Kiegerl et al. 2000). It appears that SIMK activation by SIMKK does not require an upstream MAPKKK, and SIMKK transduces both salt and pathogen elicitor stress signals (Cardinale et al. 2002). Salt stress activates *Arabidopsis* MAPKKK (ATMEKK1) and upregulates its gene expression (Ichimura et al. 1998). Co-expression of *ATMEKK1* with *MAPKKs* (*ATMKK2* and *MEK1*) complemented the growth defect of the yeast *pbs2* mutant, while co-expression of *ATMPK4* and *MEK1* complemented growth defects of the yeast *mpk1* and *bck1* mutants, suggesting that ATMEKK1, ATMKK2/MEK1, and ATMPK4 may constitute a MAP kinase cascade in *Arabidopsis* (Fig. 2; Ichimura et al. 1998).

Correlative evidence suggests that the ROS mediated signaling under salt stress occurs through MAPKs. H_2O_2 appears to act as an intermediate in ABA signaling in guard cells (Pei et al. 2000). H_2O_2 induced oxidation of Cys residues of proteins may bring conformational changes in signaling intermediates. In *Arabidopsis*, H_2O_2 induces the expression of genes involved in signaling such as calmodulin, CDPKs, His kinase, Tyr phosphatase, putative protein kinases such as ATMPK3, and genes involved in transcriptional activation such as Zn finger proteins, heat shock transcription factor, *DREB2A*, RING Zn finger protein, myb-related transcription factor, etc. (Desikan et al. 2001). ATMPK6 is activated by osmotic stresses, cold, and ROS stress imposed by H_2O_2, KO_2, paraquat and 3-amino-1,2,4-triazole (a catalase inhibitor) in *Arabidopsis* (Yuasa et al. 2001). An *Arabidopsis* MAPKKK, ANP1, is activated by H_2O_2. ANP1 initiates a phosphorylation cascade involving two MAPKs, AtMPK3 and AtMPK6. Expression of a constitutively active tobacco ANP1 orthologue, NPK1, in transgenic tobacco provided enhanced tolerance to multiple environmental stress conditions including salinity, suggesting that ANP1/NPK1 is involved in oxidative stress signaling under abiotic stresses (Fig. 2; Kovtun et al. 2000). Constitutively active ANP1 activates the MAPK cascade that activates promoters of stress-responsive genes such as *GST6* and *HSP* but not *RD29A* (Kovtun et al. 2000). This indicates that ANP1/NPK1 does not regulate DREB1, DREB2, and bZIP transcription factors, which are transcriptional activators of *RD29A* and other *COR* genes.

Nucleoside Diphosphate Kinase participates in hormone-dependent signal transduction pathways by activating guanine nucleotide-binding proteins involved in regulation of cell growth and differentiation. Transgenic analysis of NDPK2 (Nucleoside Diphosphate Kinase2) suggests that MAPK signaling regulates the

oxidative stress management and growth regulation under abiotic stresses. The *NDPK2* gene in *Arabidopsis* (*AtNDPK2*) is induced by H_2O_2. Transgenic plants overexpressing *AtNDPK2* accumulated lower levels of ROS, while *AtNDPK2* mutants accumulated higher levels of ROS than wild type. AtNDPK2 interacts with ATMPK3 and ATMPK6. These two MAPKs are activated by H_2O_2 but this response was drastically reduced in an *atndpk2* mutant. Transgenic *Arabidopsis* overexpressing *AtNDPK2* showed an enhanced tolerance to multiple environmental stresses that elicit ROS accumulation, suggests that AtNDPK2 may positively regulate H_2O_2-mediated MAPK signaling in plants (Moon et al. 2003).

MAPKs can be inactivated by dephosphorylation. *Arabidopsis* phosphotyrosine phosphatase (AtPTP1) inactivates ATMPK4 *in vitro* (Huang et al. 2000). The *AtPTP1* gene is regulated by abiotic stresses such as drought, heat shock, wounding, high salt, and cold temperature. High salt conditions increased the expression level of *AtPTP1*, while cold significantly downregulates the *AtPTP1* gene (Xu et al. 1998). The *Arabidopsis mkp1* mutant is resistant to salinity but hypersensitive to genotoxic stress induced by UV-C and methyl methanesulphonate. *MKP1* encodes a MAPK phosphatase 1 (MKP1). A yeast two-hybrid screen showed that MKP1 could interact with three *Arabidopsis* MAPKs: MPK6, MPK3, and MPK4 and the interaction was strongest with MPK4 (Ulm et al. 2002). These three MAPKs have been implicated in salt stress signaling (Mizoguchi et al. 1996; Ichimura et al. 2000). The activity of MPK6 is regulated by MKP1 *in vivo*. Mutant analysis revealed that either *MKP1* deletion or loss of MKP1 phosphatase activity results in enhanced salt tolerance. This suggests that MKP1 is a negative regulator of salt stress signaling through MAPK, while it functions as positive regulator in genotoxic stress tolerance (Ulm et al. 2002). Microarray analysis showed an increased mRNA level of a putative Na^+/H^+-exchanger (*AT4G23700*) gene in the *mkp1* mutant under salt stress (Ulm et al. 2002), which suggests that AT4G23700 may be upregulated by a MAPK cascade that is under the negative control of MPK1. It is not known whether increased salt tolerance of the *mpk1* mutant is due to increased expression of the putative Na^+/H^+-exchanger. Overexpression of *SOS1*, a plasma membrane Na^+/H^+-exchanger (Shi et al. 2003) and *AtNHX1*, a vacuolar Na^+/H^+-exchanger (Apse et al. 1999; Zhang and Blumwald 2001; Zhang et al. 2001) resulted in enhanced salt stress tolerance. The *AT4G23700* gene is located on chromosome 4 and hence is different from *SOS1* (located on chromosome 2) and *AtNHX1* (located on chromosome 5) (Ulm et al. 2002). This suggests that a salt stress-responsive MAPK cascade in *Arabidopsis* may involve ANP1, MKK1, MPK3, 4, and/or 6, and their negative regulator MKP1(Fig. 2).

9.4 ABA-mediated salt stress signaling

ABA plays an important role in many aspects of plant growth and development from germination to seed development, and also plays a pivotal role in abiotic stress resistance. Salt stress induces ABA accumulation and the amount of the increase depends upon the tissue type. In maize, salt stress increased ABA accumu-

lation up to 10-fold in roots but only 1-fold in leaf tissues. Salt stress induced ABA accumulation appears to be due to both ionic and osmotic stresses in roots, while that in the leaf is mainly due to osmotic stress (Jia et al. 2002). Turgor loss caused by osmotic stress leads to ABA synthesis and accumulation, which in turn regulates part of the cellular response to osmotic stress under salinity. ABA regulates cell water balance through stomatal regulation and genes involved in osmolyte biosynthesis, while it imparts dehydration tolerance through LEA-like genes (Hasegawa et al. 2000; Shinozaki and Yamaguchi-Shinozaki 2000; Zhu 2002). ABA signaling for stomatal closure and gene expression is transduced through Ca^{2+} (Leung and Giraudat 1998; Schroeder et al. 2001). The importance of ABA mediated stomatal regulation in salt tolerance was revealed by the analysis of *OSM1* locus of *Arabidopsis*. Root growth of the *Arabidopsis* T-DNA insertion mutant, *osm1* (for osmotic stress–sensitive mutant), was hypersensitive to NaCl or mannitol stress. Molecular cloning revealed that *OSM1* encodes a protein similar to SNARE type mammalian syntaxins (Zhu et al. 2002). SNARE proteins are required for fusion vesicle trafficking, control membrane Ca^{2+} and Cl^-channel activity and guard cell volumes (Schroeder et al. 2001). Consistent with this, ABA-mediated guard cell function is impaired in the *osm1* mutant. *OSM1* is strongly expressed in roots and leaf guard cells. The *osm1* mutant showed enhanced wilting and decreased survival when salt or drought stress was imposed on soil grown plants. Thus, OSM1 plays a critical role in root growth and in ABA regulation of stomatal responses under osmotic stresses (Zhu et al. 2002).

Osmotic stress responsive genes and ion transporters are regulated by ABA under salt stress. ABA induces several LEA-like stress responsive proteins, which are known as RD (responsive to dehydration), ERD (early responsive to dehydration), KIN (cold inducible), and RAB (responsive to ABA). Transient expression studies in isolated protoplasts showed that IP_3 and cADPR gated calcium channels are involved in ABA induced Ca^{2+} signatures. The expression of the stress responsive genes *RD29A* and *KIN2* is activated by ABA signaling through Ca^{2+} (Wu et al. 1997). ABA induces *AtPLC1* expression. Transgenic plants expressing *AtPLC1* in antisense and sense orientation showed that ABA induced expression of *RD22*, *RD29A* and *KIN2* requires AtPLC1 but it is not sufficient for maximal induction of stress responsive genes (Sanchez and Chua 2001). The *RD29A::LUC* reporter genetic screen facilitated isolation of abiotic stress and ABA signaling mutants in *Arabidopsis* (Ishitani et al. 1997). Two of these mutants, *los5* and *los6*, were impaired in the expression of stress responsive genes, such as *RD29A*, *COR15A*, *COR47*, *RD22*, and *P5CS*, under salt and osmotic stresses. Salt induced *RD29A::LUC* expression was restored to the wild type level by exogenous application of ABA. These mutants were also defective in osmotic stress induced ABA biosynthesis. Molecular cloning revealed that *LOS5* encodes a molybdenum cofactor sulfurase, which is allelic to *ABA3* (Xiong et al. 2001a), while *LOS6* encodes zeaxanthin epoxidase, which is allelic to *ABA1* (Xiong et al. 2002a). These results demonstrate that stress responsive gene expression under salinity is mediated by ABA. Salt stress and ABA upregulate a vacuolar Na^+/H^+ antiporter, *AtNHX1*, which was reduced in ABA deficient mutants (*aba2-1* and *aba3-1*), but not in *salt overly sensitive mutants* (*sos1*, *sos2* or *sos3*) mutants. The *abi1-1* but not in *abi2-1*

mutation decreased ABA and salt-induced *AtNHX1* expression. *AtNHX1* contains putative ABRE elements between –736 to –728 from the initiation codon. These results suggest that transcriptional upregulation of *AtNHX1* under salt stress is partially dependent on ABA biosynthesis and ABA signaling through the 2C type protein phosphatase ABI1 (Fig. 2; Shi and Zhu 2002).

ABA-deficient *los5/aba3* and *los6/aba1* mutants are more tolerant to salt stress at germination but at the vegetative stage *los5/aba3* is hypersensitive to salt stress (Xiong et al. 2001a & 2002a). *Arabidopsis* T-DNA insertion mutant *sañ5 (SALOBREÑO)* is tolerant to osmotic stress (NaCl, KCl, and mannitol) and ABA during germination. This mutation is allelic to *abi4* (Quesada et al. 2000). The quantitative trait loci (QTL) for the most effective ABA response at germination were mapped very close to the QTL for salt tolerance in germination (Mano and Takeda 1997). The QTLs for salt tolerance at germination were different from those of QTLs controlling salt tolerance at the seedling stage, indicating that salt tolerance at germination and at the seedling stage are regulated by different mechanisms (Mano and Takeda 1997; Quesada et al. 2002). Salt sensitivity in germinating seeds is mainly due to inhibition of germination by salt stress-induced ABA.

9.5 The SOS signaling pathway of ion homeostasis

Cellular ion homeostasis under salinity is achieved by the following strategies: 1) Exclusion of Na^+ from the cell by plasma membrane-bound Na^+/H^+ antiporters or by limiting the Na^+ entry, 2) Utilization of Na^+ for osmotic adjustment by compartmentation of Na^+ into the vacuole through tonoplast Na^+/H^+ antiporters, and 3) Na^+ secretion. Thus, regulation of ion transport systems is fundamental to plant salt tolerance. Genetic analysis of *s*alt *o*verly *s*ensitive (*sos*) mutants of *Arabidopsis*, led to the identification of the SOS pathway, which regulates cellular ion homeostasis and salt tolerance (Fig. 1; Zhu 2002).

The *Arabidopsis sos3* mutant is hypersensitive to salt stress. Molecular cloning revealed that the *SOS3* encodes a Ca^{2+} binding protein homologous to the regulatory subunit of yeast calcineurin and animal neuronal calcium sensors. It has an N-myristoylation motif and three calcium binding EF hands. SOS3 senses salt stress-induced increases in cytosolic Ca^{2+} concentration in plants (Liu and Zhu 1998; Ishitani et al. 2000). Myristoylated SOS3 is recruited to the plasma membrane (Quintero et al. 2002). Mutations that disrupt either myristoylation (G2A) or calcium binding (*sos3-1*) cause salt stress hypersensitivity to *Arabidopsis* plants. Since myristoylation of SOS3 is essential for salt tolerance, it is likely that membrane recruitment of SOS3 is essential for its function. Membrane localization of SOS3 may help in the regulation of its target ion transporters (Ishitani et al. 2000).

Identification of additional *SOS* loci (*SOS2* and *SOS1*) revealed that the SOS pathway regulates cellular ion homeostasis under salt stress. *Arabidopsis sos1* and *sos2* mutants are also hypersensitive to salt stress and *sos1*, *sos2*, and *sos3* mutations do not show an additive effect, implying that they are in the same pathway of

salt stress response. SOS2 is a ser/thr protein kinase with an N-terminal kinase catalytic domain and a C-terminal regulatory domain. The SOS2 C-terminal regulatory domain consists of the SOS3-binding, autoinhibitory FISL motif (Liu et al. 2000). Binding of SOS3 activates the SOS2 protein kinase (Halfter et al. 2000). Deletion of the FISL motif from SOS2 leads to constitutive activation of the kinase (Guo et al. 2001). Molecular genetic analysis of the *sos1* mutant led to the identification of a target for the SOS3-SOS2 kinase complex. *SOS1* encodes a plasma membrane Na^+/H^+ antiporter (Shi et al. 2000). The *sos1* mutant accumulates high levels of Na^+ in tissues under salt stress, and isolated plasma membrane vesicles from *sos1* mutants showed significantly less Na^+/H^+ exchange activity than the wild type, suggesting that the SOS1 Na^+/H^+ antiporter is located on the plasma membrane (Qiu et al. 2002). The *sos3* and *sos2* mutants accumulate higher levels of Na^+ than wild type plants. Isolated plasma membranes vesicles from these mutants also showed significantly less Na^+/H^+ exchange activity, and this could be restored to the wild type levels by the addition of activated SOS2. The SOS3-SOS2 kinase complex activates SOS1 by phosphorylation (Quintero et al. 2002). *SOS1* complemented yeast mutants defective in Na^+ transporters. Co-expression of *SOS2* and *SOS3* significantly increased *SOS1*-dependent Na^+ tolerance of the yeast mutant (Quintero et al. 2002). These results show that SOS1 is a Na^+/H^+ antiporter involved in Na^+ efflux, which is activated by the SOS3-SOS2 kinase complex (Fig.1 1; Qiu et al. 2002; Quintero et al. 2002). Constitutive expression of a CaMV 35S promoter driven active form of SOS2 could rescue *sos2* and *sos3* mutants under salt stress (Xiong et al. 2002b).

The expression of *SOS1* is stronger in cells bordering the xylem. Under salt stress (100 mM NaCl), a higher concentration of Na^+ accumulates in shoots of *sos1* mutants than in those of the wild type. These results suggest that SOS1 might retrieve Na^+ from the xylem, thereby preventing excess Na^+ accumulation in the shoot (Shi et al. 2002a). Transgenic *Arabidopsis* plants overexpressing *SOS1* showed improved salt tolerance and accumulated less Na^+ in the xylem transpirational stream as well as in the shoot compared to the wild type plants. This demonstrated that Na^+ efflux from the root cells and long distance Na^+ transport within the plant under salt stress are regulated by SOS1 (Shi et al. 2003), which in turn is regulated by the SOS3-SOS2 kinase complex. In addition to the activation of Na^+/H^+ antiporter activity of SOS1, SOS3-SOS2 kinase complex also is involved in salt stress induced upregulation of *SOS1* expression (Fig. 1; Shi et al. 2000). In the *sos3* mutant salt, stress could not induce *SOS1* expression, while the *sos2* mutant is impaired in *SOS1* expression only in roots, but not in shoots. Interestingly, *SOS1* overexpressing transgenic *Arabidopsis* showed a significantly higher steady state level of *SOS1* mRNA under salt stress than that grown under normal conditions. Since *SOS1* was overexpressed under the control of the CaMV 35S promoter, its higher mRNA abundance under salt stress might be due to an increase in *SOS1* transcript stability (Shi et al. 2003).

In addition to positive control of Na^+ exclusion from the cytosol, the SOS pathway may also negatively regulate Na^+ influx systems. Expression of plant high affinity K^+ transporters, *AtHKT1*, *EcHKT1*, and *EcHKT2*, in *Xenopus laevis* oocytes showed that they could mediate Na^+ uptake. Transgenic wheat plants ex-

pressing the wheat *HKT1* in antisense orientation under control of a ubiquitin promoter showed significant downregulation of the native *HKT1* transcript. These lines showed significantly less ^{22}Na uptake and enhanced growth under salinity when compared with the control (Laurie et al. 2002). These results suggest that HKT1 mediates sodium uptake under salinity, and salt tolerance can be improved by downregulation of *HKT1* expression. Consistent with this observation, a suppressor genetic screen for the *sos3* mutation revealed that functional disruption of *AtHKT1* could suppress the salt-sensitive phenotype of *sos3*. In addition, the *athkt1* mutation alleviates the K^+-deficient phenotype of the *sos3* mutant (Rus et al. 2001a), which suggests that the K^+-deficient phenotype of the *sos3* mutant might be due to an excess of cytoplasmic Na^+, as *sos3* impairs the Na^+ efflux mediated by SOS1. These results suggest that ATHKT1 might function as low affinity Na^+ transporter that is involved in Na^+ influx under salinity. Significant amounts of Na^+ enter plant roots through voltage independent channels, which are probably regulated by Ca^{2+} concentrations (Tyerman and Skerret 1999). We do not know whether activity of these channels and their gene expression are also regulated by the calcium dependent SOS3-SOS2 kinase complex. Thus, the SOS3-SOS2 kinase complex positively regulates Na^+ efflux by activating SOS1 and upregulating the *SOS1* transcript level, and may negatively regulate Na^+ influx by downregulating low affinity Na^+ transporter (*HKT1*) genes to restore cellular ion homeostasis under salt stress in plants (Fig. 1; Zhu 2002)

9.6 Osmotic stress management

Plant survival depends on maintaining a positive turgor, which is indispensable for expansion growth of cells and stomatal opening. A decrease in water availability under soil salinity causes osmotic stress, which leads to decreased turgor. Osmotic adjustment is one of the vital cellular tolerance process to osmotic stress, conserved in both halophytic and glycophytic plants. Osmotic stress may induce ion (Na^+ & K^+) uptake and compartmentalization into the vacuole, and synthesis of organic compatible solutes such as proline, betaine, polyols, and soluble sugars. Use of ions for osmotic adjustment may be energetically more favorable than organic osmolyte biosynthesis under stress, as ion uptake and sequestration into the vacuole may cost only 3-4 moles of ATP compared with the 30-50 moles of ATP needed for synthesis of one mole of organic osmolytes (Raven 1985).

9.6.1 Sodium sequestration into the vacuole

Cytoplasmic ion homeostasis by exclusion of excess Na^+ from the cytoplasm may necessitate the plant to synthesize compatible osmolytes to reduce the osmotic potential, which is required for water uptake under salt stress. Hence, compartmentation of Na^+ in the vacuole is an important strategy for plants, to maintain a lower Na^+ concentration at the sites of biochemical reactions in the cytosol, and yet

maintain a lower overall osmotic potential. Active transport of solutes across biological membranes utilizes the electrochemical gradient generated by P-type H^+-ATPases (plasma membrane H^+-ATPases), V-type H^+-ATPases (vacuolar H^+-ATPase) and H^+-pyrophosphatase (vacuolar H^+-PPase). The sodium efflux plasma membrane Na^+/H^+ antiporters use a proton electrochemical gradient generated by the plasma membrane H^+-ATPase, which is upregulated under salinity. A salt-tolerant mutant of rice showed higher induction of the plasma membrane H^+-ATPase gene *OSA3* in roots than that of the wild type (Zhang et al. 1999). Influx of Na^+ into the vacuole occurs through Na^+/H^+ antiporters, which use the proton gradient generated by V-type H^+-ATPase and H^+-PPase (Apse et al. 1999). Thus, Na^+ sequestration into the vacuole depends upon the expression and activity of Na^+/H^+ antiporters as well as V-type H^+-ATPase and H^+-PPase. Salinity upregulates the expression of a V-type H^+-ATPase gene (Golldack and Dietz 2001) and a vacuolar Na^+/H^+ antiporter gene (Gaxiola et al. 1999; Shi and Zhu 2002). To investigate the role of tonoplast H^+-PPase in salinity tolerance, the *AVP1* gene (vacuolar H^+-pyrophosphatase) was overexpressed in *Arabidopsis*. The transgenics showed increased sequestration of Na^+ into the vacuole, maintained higher relative water content in leaves and were more tolerant to salt and drought stress than the wild type was (Gaxiola et al. 2001).

In *Arabidopsis*, the *AtNHX1* gene encodes a tonoplast Na^+/H^+ antiporter. Expression of *AtNHX1* in the yeast *nhx1* mutant suppressed some of the mutant phenotypes. Salinity induces *NHX1* expression in *Arabidopsis* (Gaxiola et al. 1999; Shi and Zhu 2002) and rice (Fukuda et al. 1999). Transgenic *Arabidopsis* plants that overexpress *AtNHX1* showed significantly higher salt tolerance than wild type plants (Apse et al. 1999). Similarly, transgenic tomato and canola (*Brassica napus*) plants overexpressing *AtNHX1* accumulated high concentrations of sodium in leaves but not in fruits/seeds. These transgenics were shown to be highly tolerant to salt stress at the same time they maintained the quality of fruit in tomato and oil in canola (Zhang and Blumwald 2001; Zhang et al. 2001). These studies confirm that sequestration of Na^+ into the vacuole is an important trait of salt tolerance in plants.

9.6.2 K^+ Uptake

Plants maintain a high cytosolic K^+/Na^+ ratio under optimal conditions. Salt stress induced decrease in the K^+/Na^+ ratio is inimical to cellular biochemical processes. In addition to this, K^+ provides necessary osmotic potential for water uptake by plant cells (Keller and Van Volkenburgh 1996; Claussen et al. 1997). Thus, K^+ uptake is pivotal for cell turgor and maintenance of biochemical processes under salinity. In plants, Na^+ competes with K^+ for uptake under saline conditions. The *Mesembryanthemum crystallinum* K^+ transporter genes, *McHAK1* and *McHAK2*, are upregulated under K^+ starvation and NaCl stress in both roots and leaves (Su et al. 2002). Low K^+ concentration in the growth medium inhibits the growth of *sos* mutants. The *sos3* mutant could be rescued by increasing Ca^{2+} in a low K^+ me-

dium (Zhu et al. 1998). Hence, expression of transport systems specific for K^+ uptake might help in maintaining ionic balance.

Overexpression of *AtHAL3a* (a regulator of K^+ transport) in yeast and *Arabidopsis* conferred increased salt tolerance (Espinosa-Ruiz et al. 1999), as did transgenic melon plants expressing the *HAL1* gene (Bordás et al. 1997). To investigate the role of HAL1 *in vivo*, tomato plants were engineered to overexpress the yeast *HAL1* gene. This transgenic plant showed increased K^+ accumulation under NaCl stress (Rus et al. 2001b). Transgenics showed better salt tolerance than the control plants (Gisbert et al. 2000; Rus et al. 2001b), suggesting that K^+ accumulation is an important trait of salt tolerance. Further, the *Arabidopsis sos4* mutant defective in the pyridoxal kinase gene showed hypersensitive-root growth under NaCl and KCl stresses and accumulated more Na^+ but less K^+. Pyridoxal-5-phosphate and its derivatives act as ligands for P2X receptor ion channels in animals. ATP is required for K^+ channel activity and a cyclic nucleotide-binding site is required for K^+ channel (KAT1) function. Thus regulation of K^+ and Na^+ channels or transporters by pyridoxal-5-phosphate and its derivatives may be important in plant salt tolerance (Table 1; Shi et al. 2002b)

9.6.3 Osmoprotectant biosynthesis

Organic compatible solutes/osmoprotectants protect plants from stress by (1) osmotic adjustment which helps in turgor maintenance (2) detoxification of reactive oxygen species and (3) stabilization of the quaternary structure of proteins (Yancey et al. 1982; Bohnert and Jensen 1996). Genes involved in osmoprotectant biosynthesis are upregulated under salt and drought stresses (Zhu 2002; Xiong et al. 2001a). Enhanced tolerance to salt stress was observed in transgenic plants engineered to over-accumulate mannitol (Tarczynski et al. 1993; Karakas et al. 1997; Sheveleva et al. 1997; Shen et al. 1997), glycine betaine (Holmstrom et al. 2000; Hayashi et al. 1997; Sakamoto et al. 1998; Kishitani et al. 2000; Prasad et al. 2000), and proline (Kishor et al. 1995; Zhu et al. 1998; Nanjo et al. 1999; Hong et al. 2000). Transgenic rice plants expressing a peroxisomal betaine aldehyde dehydrogenase of barley accumulated fewer Na^+ and Cl^- ions and more K^+ ions (Kishitani et al. 2000).

Further evidence for the involvement of osmoprotectants in salt tolerance came from analysis of the *Arabidopsis* mutant, *t365*, in which the S-adenosyl-L-methionine phosphoethanolamine N-methyltransferase (*PEAMT*) gene is silenced (Table 1). The PEAMT protein catalyzes all three methylation steps required to convert phosphoethanolamine to phosphocholine, which is a precursor of choline biosynthesis. Some plants synthesize the osmoprotectant glycinebetaine from choline. The *t365* mutants produced significantly less choline and showed hypersensitivity to salinity in addition to temperature-sensitive male sterility (Mou et al. 2002), which supports the importance of osmoprotectant in salt tolerance. The ectopic expression studies showed that osmoprotectants increase salt stress tolerance mainly by protection of membranes and proteins against reactive oxygen species (ROS) rather than by increasing osmotic adjustment. ABA regulates the *P5CS*

gene involved in proline biosynthesis under osmotic stress (Xiong et al. 2001a). A signaling cascade similar to that of the yeast MAPK HOG1 pathway may also regulate osmolyte biosynthesis.

9.7 Stress damage control and repair

9.7.1 Salt stress induced proteins

In higher plants, osmotic stress induces several proteins in vegetative tissues, which are related to late-embryogenesis-abundant (LEA) proteins. The correlation between LEA protein accumulation in vegetative tissues and stress tolerance in various plant species indicates its protective role under dehydration stress (reviewed by Ingram & Bartels 1996). Engineered rice plants overexpressing a barley *LEA* gene, *HVA1*, under control of the rice actin 1 promoter showed better stress tolerance under 200 mM NaCl and drought stress than did the wild type (Xu et al. 1996). *Arabidopsis* LEA-like stress proteins are encoded by *COR* genes (*RD29A, COR47, COR15, KIN1, KIN2*) which are induced by cold, dehydration (due to water deficit or high salt), or ABA. Promoter analysis of the *COR* genes showed that many of them contain dehydration responsive elements (DRE) or C-Repeat (CRT), as well as ABA-responsive elements or ABREs. Transcription factors that regulate the LEA-like genes include CBFs (C-repeat Binding Proteins, also known as Dehydration Responsive Element Binding Proteins, DREBs) and bZIP proteins. The expression of *COR* genes is regulated by both ABA dependent and independent pathways (Ishitani et al. 1997; Shinozaki and Yamaguchi-Shinozaki 2000). Constitutive overexpression of *CBF3* or stress induced expression of *CBF3* driven by the *RD29A* promoter resulted in enhanced expression of *COR* genes under cold, dehydration, and salt stresses in transgenic *Arabidopsis* and also conferred higher osmotic stress tolerance (Kasuga et al. 1999). *CBF3*-overexpression in *Arabidopsis* also resulted in elevated accumulation of proline and total soluble sugars, including sucrose, raffinose, glucose, and fructose. The increase in proline levels was due to increased expression of the key proline biosynthetic enzyme Δ^1-pyrroline-5-carboxylate synthase (Gilmour et al. 2000). Thus, LEA-like proteins appear to protect plants under salt stress. Osmotic or salt stress-induced calcium signals may activate the LEA-like genes through DREB2 transcription factors, while salt stress induced ABA accumulation appears to induce the genes through ABA responsive element binding factors (Xiong et al. 2002b; Zhu 2002).

The *Alfin1* gene of *Medicago sativa* encodes a member of the zinc-finger family transcription factors, and its expression is correlated with NaCl tolerance (Winicov and Bastola 1999; Winicov 2000). *In vitro*, Alfin1 binds to the promoter of *MsPRP2*, which encodes a salt stress inducible root-specific cell wall protein. The *Alfin1* gene appears to be conserved in alfalfa, rice, and *Arabidopsis*. The role of *Alfin1* in salt stress tolerance was examined in transgenic alfalfa expressing *Alfin1* driven by the *CaMV 35S* promoter in the sense and antisense orientations. Although overexpression lines did not show any growth defect, the antisense trans-

genic plants grew poorly in soil in a normal environment, demonstrating that *Alfin1* expression is essential for normal plant development. *Alfin1* overexpression enhanced the root growth significantly both under normal and saline conditions, while the antisense plants showed poor root growth (Winicov and Bastola 1999; Winicov 2000). The tobacco-stress-induced-gene 1 (*Tsi1*) encodes a DNA-binding protein with an EREBP/AP2 DNA binding motif, which is involved in defense- and drought-responsive gene expression. *Tsi1* gene expression was rapidly induced by salt stress but not by drought or ABA. Overexpression of *TSI1* in tobacco enhanced retention of chlorophyll content when the leaves were floated on 400 mM NaCl solution for 48 or 72 hr (Park et al. 2001). Further studies are needed to assess the role of Alfin1 and TSI1 in salt stress tolerance, as it is not clear at present whether these proteins and their targets are involved in ion/osmotic homeostasis or in detoxification.

9.8 Oxidative stress management

Reactive oxygen species (ROS) namely, superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) are produced in aerobic cellular processes such as mitochondrial and chloroplast electron transport, or oxidation of glycolate (photorespiration), xanthine, and glucose. Due to metabolic disturbance under stress conditions, ROS production increases under abiotic stresses including salinity (Smirnoff 1993; Gomez et al. 1999; Hernandez et al. 2001). The ROS causes oxidative damage to membrane lipids, proteins and nucleic acids. Hence, ROS detoxification forms an important defense against abiotic stresses. The antioxidants employed by plants are ascorbate, glutathione, α-tocopherol, and carotenoids. Detoxifying enzymes include superoxide dismutase (SOD), catalase, and enzymes of the ascorbate- glutathione cycle. The *Arabidopsis* salt tolerant mutant *pst1* (for *photoautotrophic salt tolerance1*) is more tolerant to oxidative stress than is the wild type (Table 1). The *pst1* mutant did not differ in proline accumulation or monovalent cation (sodium, potassium) accumulation when compared to the wild type. Under salt stress, the *pst1* mutant showed significantly higher activity of superoxide dismutase and ascorbate peroxidase than that of wild type *Arabidopsis* (Tsugane et al. 1999). Overexpressing the tobacco *NtGST/GPX* gene (encoding an enzyme with both glutathione S-transferase and glutathione peroxidase activity) in transgenic tobacco plants improved salt and chilling stress tolerance due to enhanced ROS scavenging and prevention of membrane damage (Roxas et al. 1997; Roxas et al. 2000). Transgenic tobacco plants expressing the constitutively active MAPKKK, ANP1, show an activated MAPK cascade that activates the glutathione S-transferase 6 (*GST6*) gene promoter. These transgenic plants were also tolerant to salt and other abiotic stresses (Kovtun et al. 2000). Components of MAPK cascades are activated by ROS and salinity as discussed earlier. Thus, it appears ROS management under salt stress through the induction of genes encoding antioxidant enzymes may be controlled by a MAPK signaling cascade (Fig. 2).

9.9 Growth regulation

Maintenance of root growth at low water potential is an adaptive trait of osmotic stress tolerance. In maize roots, salt stress increased ABA accumulation up to 10-fold (Jia et al. 2002). Root elongation at low water potential might be achieved by an increase in the activity of the putative wall loosening enzyme xyloglucan endotransglycosylase (Wu et al. 1994) and proline accumulation (Ober and Sharp 1994), which are regulated by ABA.

Root elongation at low water potential was impaired in the *vp5* mutant, or by a chemical inhibitor of ABA biosynthesis (fluridone) in maize. However, this could be restored by treatment of roots with a chemical that inhibits ethylene biosynthesis or action. Moreover, treatment of seedlings with fluridone resulted in an increase in the rate of ethylene production. These data suggested that ABA-mediated root cell elongation under osmotic stress might be due to its inhibition of ethylene biosynthesis (Spollen et al. 2000).

In *Arabidopsis*, a null allele of the Gα gene impaired cell division (Ullah et al. 2001) and ABA inhibition of stomatal closure (Wang et al. 2001). A loss-of-function allele of another ABA signaling locus encoding a SNARE protein, *osm1*, also showed impaired root growth under salt and osmotic stress (Zhu et al. 2002). This suggests that ABA may regulate cell division under osmotic stress. Consistent with this, the transcripts of a cyclin-dependent kinase (*AtCDC2a*) and two mitotic cyclin (*AtCycB1* and *AtCycA2*) genes were diminished initially but induced subsequently in the shoot apex during salt stress adaptation (Burssens et al. 2000). SIMK is activated and translocated into the nucleus in suspension-cultured alfalfa cells under salt stress (Baluska et al. 2000). In the root elongation zone, epidermal cells contained much higher SIMK protein than in cortex cells. SIMK showed a cell cycle phase-dependent localization, being predominantly nuclear in interphase but associating with the cell plate and the newly formed cell wall in telophase and early G1 phase (Baluska et al. 2000). It is not clear whether cell division/elongation is regulated through MAPK signaling under salt stress.

In the root tips of *Arabidopsis*, *AtCDC2a*, *AtCycA2* and *AtCycB1* expression were diminished concomitant with inhibition of root growth under salt stress (Burssens et al. 2000). The activity of CDC2a is negatively regulated by a cyclin-dependent protein kinase inhibitor, ICK1. The expression of *ICK1* is upregulated by ABA in *Arabidopsis* (Wang et al. 1998). The knowledge of tissue- and plant species-specific regulation of cell division/elongation by ABA under salt stress is still in its infancy.

9.10 Conclusions and perspectives

Although a salt stress sensor is yet to be identified, some of the components of salt stress signaling and plant salt tolerance are known today. Genetic evidence demonstrated that a salt stress induced calcium signal is transduced at least in part through the SOS3-SOS2 kinase complex, which activate SOS1, a plasma mem-

brane Na^+/H^+ antiporter. In addition, the SOS3-SOS2 kinase complex positively regulates the *SOS1* transcript level. Correlative evidence implicates the involvement of a putative osmosensory histidine kinase (AtHK1) and a MAPK cascade in osmoprotectant biosynthesis under salt stress. Responses to ion toxicity, osmotic stress and oxidative stress may be integrated by signaling pathways including MAPK and its negative regulator MAPK phosphatase. Except for the SOS pathway, salt stress signaling pathways are not yet understood in terms of their components and targets. Moreover, the evidence summarized here is mainly derived from studying *Arabidopsis*, a glycophytic plant and hence further analysis of salt tolerance mechanisms in halophytic plants is also warranted. Characterization of chloride and sulfate transporters and their regulation under salt stress is also the need of the hour. Salt tolerance varies with plant development, and it is imperative to understand the tissue and developmental specificity of salt stress tolerance. Availability of whole genome sequences in *Arabidopsis* and rice, as well as the use of microarrays to analyze the transcriptome response will facilitate the identification of genes involved in salt tolerance, which can be validated by RNA interference and/or T-DNA/transposon/EMS mutational studies. Continued genetic and biochemical dissection of salt tolerance in the near future may provide us a clear picture of salt tolerance in plants, which will help to engineer agronomically useful salt tolerant crop varieties.

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The cover illustration depicts pseudohyphal filaments of the ascomycete *Saccharomyces cerevisiae* that enable this organism to forage for nutrients. Pseudohyphal filaments were induced here in a wild-type haploid MATa Σ1278b strain by an unknown readily diffusible factor provided by growth in confrontation with an isogenic petite yeast strain in a sealed petri dish for two weeks and photographed at 100X magnification (provided by Xuewen Pan and Joseph Heitman).

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