

Understanding and Improving Salt Tolerance in Plants

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ABSTRACT

One-fifth of irrigated agriculture is adversely affected by soil salinity. Hence, developing salt-tolerant crops is essential for sustaining food production. Progress in breeding for salt-tolerant crops has been hampered by the lack of understanding of the molecular basis of salt tolerance and lack of availability of genes that confer salt tolerance. Genetic evidence suggests that perception of salt stress leads to a cytosolic calcium-signal that activates the calcium sensor protein SOS3. SOS3 binds to and activates a ser/thr protein kinase SOS2. The activated SOS2 kinase regulates activities of SOS1, a plasma membrane Na⁺/H⁺ antiporter, and NHX1, a tonoplast Na⁺/H⁺ antiporter. This results in Na⁺ efflux and vacuolar compartmentation. A putative osmosensory histidine kinase (AtHK1)-MAPK cascade probably regulates osmotic homeostasis and ROS scavenging. Osmotic stress and ABA (abscisic acid)-mediated regulation of LEA (late-embryogenesis-abundant)-type proteins also play important roles in plant salt tolerance. Genetic engineering of ion transporters and their regulators, and of the CBF (C-repeat-binding factor) regulators, holds promise for future development of salt-tolerant crops.

SALINITY is one of the major abiotic stresses that adversely affect crop productivity and quality. About 20% of irrigated agricultural land is adversely affected by salinity (Flowers and Yeo, 1995). The problem of soil salinity is further increasing because of the use of poor quality water for irrigation and poor drainage. In clay soils, improper management of salinity may lead to soil sodicity whereby sodium binds to negatively charged clay causing clay swelling and dispersal that makes the soil less fit for crop growth. According to the USDA salinity laboratory, saline soil can be defined as soil having an electrical conductivity of the saturated paste extract (EC_e) of 4 dS m⁻¹ (4 dS m⁻¹ ≈ 40 mM NaCl) or more. Most grain crops and vegetables are glycophytes and are highly susceptible to soil salinity even when the soil EC_e is <4 dS m⁻¹. Different threshold tolerance EC_e and different rate of reduction in yield beyond threshold tolerance EC_e indicate variation in mechanisms of salt tolerance among crop species (Table 1).

Soil type and environmental factors, such as vapor pressure deficit, radiation, and temperature may further alter salt tolerance. Adverse effects of salinity on plant growth may be due to ion cytotoxicity (mainly due to Na⁺, Cl⁻, and SO₄²⁻), and osmotic stress (reviewed by Zhu, 2002). Most crop plants are susceptible to salinity

even when EC_e is <3.0 dS m⁻¹ (Table 1), which in terms of osmotic potential is less than -0.117 MPa (osmotic potential = -0.39 × EC_e). At these salinity levels, the predominant cause of crop susceptibility appears to be ion toxicity rather than osmotic stress. Ion cytotoxicity is caused by replacement of K⁺ by Na⁺ in biochemical reactions and conformational changes and loss of function of proteins as Na⁺ and Cl⁻ ions penetrate the hydration shells and interfere with noncovalent interactions between their amino acids. Metabolic imbalances caused by ionic toxicity, osmotic stress, and nutritional deficiency under salinity may also lead to oxidative stress (Zhu, 2002). Hence, engineering crops that are resistant to salinity stress is critical for sustaining food production and achieving future food security. Understanding the molecular basis of salt-stress signaling and tolerance mechanisms is essential for breeding and genetic engineering of salt tolerance in crop plants. Here, we discuss the molecular basis of cellular ion homeostasis, osmotic homeostasis, stress damage control and repair under salt stress, and their exploitation for genetic engineering of salt-tolerant crop plants.

Sensors of Salt Stress

Plants sense salt stress through both ionic (Na⁺) and osmotic stress signals. Excess Na⁺ can be sensed either on the surface of the plasma membrane by a transmembrane protein or within the cell by membrane proteins or Na⁺ sensitive enzymes (Zhu, 2003). In addition to its role as an antiporter, the plasma membrane Na⁺/H⁺ antiporter SOS1 (*S*alt *O*verly *S*ensitive 1), having 10 to 12 transmembrane domains and a long cytoplasmic tail, may act as a Na⁺ sensor (Zhu, 2003). This dual role would be analogous to the sugar permease BglF in *Escherichia coli* and the yeast ammonium transporter Mep2p. When expressed in *Xenopus laevis* oocytes Na⁺-K⁺ cotransporters from *Eucalyptus camaldulensis* Dehnh. show increased ion uptake under hypoosmotic conditions while, their *Arabidopsis* homolog do not show this osmosensing capacity (Liu et al., 2001). Entry of Na⁺ through nonspecific ion channels under salinity may cause membrane depolarization that activates Ca²⁺ channels (Sanders et al., 1999), and thus generates Ca²⁺ oscillations, and signals salt stress. Cell volume decreases because of turgor loss under salinity-induced hyperosmotic stress may lead to retraction of the plasma membrane from the cell wall, which is probably sensed by both stretch-activated channels and transmembrane protein kinases, such as two component histidine kinases and wall-associated kinases (Urao et al., 1999; Kreps et al., 2002; Seki et al., 2002). Salinity up-regulates the biosynthesis of the plant stress hormone ABA (Jia et al., 2002; Xiong and Zhu, 2003), and causes accumulation of reactive oxygen species (ROS) (Smirnoff, 1993; Hernandez et al., 2001). ABA and ROS also regulate ionic and osmotic

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Table 1. Many important crops are susceptible to soil salinity† (Maas, 1990).

Crop	Threshold salinity	Decrease in yield
	dS m ⁻¹	Slope % per dS m ⁻¹
Bean (<i>Phaseolus vulgaris</i> L.)	1.0	19.0
Eggplant (<i>Solanum melongena</i> L.)	1.1	6.9
Onion (<i>Allium cepa</i> L.)	1.2	16.0
Pepper (<i>Capsicum annuum</i> L.)	1.5	14.0
Corn (<i>Zea mays</i> L.)	1.7	12.0
Sugarcane (<i>Saccharum officinarum</i> L.)	1.7	5.9
Potato (<i>Solanum tuberosum</i> L.)	1.7	12.0
Cabbage (<i>Brassica oleracea</i> var. <i>capitata</i> L.)	1.8	9.7
Tomato (<i>Lycopersicon esculentum</i> Mill.)	2.5	9.9
Rice, paddy (<i>Oryza sativa</i> L.)	3.0	12.0
Peanut (<i>Arachis hypogaea</i> L.)	3.2	29.0
Soybean [<i>Glycine max</i> (L.) Merr.]	5.0	20.0
Wheat (<i>Triticum aestivum</i> L.)	6.0	7.1
Sugar beet (<i>Beta vulgaris</i> L.)	7.0	5.9
Cotton (<i>Gossypium hirsutum</i> L.)	7.7	5.2
Barley (<i>Hordeum vulgare</i> L.)	8.0	5.0

† Lack of a direct correlation between the threshold salinity and yield decrease per unit increase in salinity may be attributed to the differences in salt exclusion, uptake, compartmentation and other mechanisms of salt tolerance among these crop species.

homeostasis as well as stress damage control and repair processes.

Regulation of K⁺ uptake and/or prevention of Na⁺ entry, efflux of Na⁺ from the cell, and utilization of Na⁺ for osmotic adjustment are strategies commonly used by plants to maintain desirable K⁺/Na⁺ ratios in the cytosol. Osmotic homeostasis is established either by Na⁺ compartmentation into the vacuole or by biosynthesis and accumulation of compatible solutes. ROS detoxification systems as well as stress proteins belonging to the LEA protein family contribute to prevention of salt-stress damage (Zhu, 2002). In addition to these mechanisms, Na⁺ secretion is a strategy used by some halophytic plants. Thus, precise regulation of ion transport systems is critical for salt tolerance. Important insights into ion homeostasis under salt stress have emerged from the molecular genetic analysis of salt overly sensitive (*sos*) mutants of *Arabidopsis* (Fig. 1; Zhu, 2003).

Sodium Influx and K⁺/Na⁺ Balance

A high K⁺/Na⁺ ratio in the cytosol is essential for normal cellular functions of plants. Na⁺ competes with K⁺ uptake through Na⁺-K⁺ cotransporters, and may also block the K⁺-specific transporters of root cells under salinity (Zhu, 2003). This results in toxic levels of sodium as well as insufficient K⁺ concentration for enzymatic reactions and osmotic adjustment. Under salinity, sodium gains entry into root cell cytosol through cation channels or transporters (selective and nonselective) or into the root xylem stream via an apoplastic pathway depending on the plant species. In *Arabidopsis* (Uozumi et al., 2000), *Eucalyptus* (Liu et al., 2001), and wheat, it has been shown that high affinity K⁺ transporters (HKT) act as low affinity Na⁺ transporters (Rubio et al., 1995; Gorham et al., 1997) under salinity. The HKT transporters of *Eucalyptus camaldulensis* are more permeable to Na⁺ than they are to K⁺ when extracellular concentrations of Na⁺ and K⁺ are equal (Liu et al., 2001). Hence, under salinity HKT homologs may contribute to Na⁺ influx. However, in rice, sodium influx into the xylem through the apoplastic pathway appears to be more significant (Yadav et al., 1996; Garcia et al.,

1997). Silica deposition and polymerization of silicate in the endodermis and rhizodermis blocks Na⁺ influx through the apoplastic pathway in roots of rice (Yeo et al., 1999). Restriction of sodium influx either into the root cells or into the xylem stream is one way of maintaining the optimum cytosolic K⁺/Na⁺ ratio of plants under salt stress. The *hkt1* mutation suppresses the salt hypersensitivity and K⁺-deficient phenotype of the *Arabidopsis* Salt Overly Sensitive 3 (*sos3*) mutant (Rus et al., 2001a). Antisense expression of wheat *HKT1* in transgenic wheat causes significantly less ²²Na uptake and enhances growth under salinity when compared with control plants (Laurie et al., 2002). These results suggest that either inactivation of the low affinity Na⁺ transporter (HKT) activity or suppression of its expression can considerably improve plant salt tolerance.

In saline conditions, cellular potassium levels can be maintained by activity or expression of potassium-specific transporters. In *Mesembryanthemum crystallinum* L., high affinity K⁺ transporter-K⁺ uptake genes are up-regulated under NaCl stress (Su et al., 2002). In yeast, *HAL1* and *HAL3* regulate K⁺ uptake and Na⁺ efflux. Overexpression of the *Arabidopsis HAL3a* gene enhances salt tolerance of transgenic *Arabidopsis* (Espinoso-Ruiz et al., 1999). Similarly, transgenic tomato plants overexpressing yeast *HAL1* gene show a higher K⁺/Na⁺ ratio and improved salt tolerance than control plants. Transgenic tomato plants exhibit lower reduction in fruit yield than that of control plants when irrigated with 35 mM NaCl (Rus et al., 2001b). Signaling events that regulate the potassium-specific transporters under salinity should be understood.

Sodium Efflux

Sodium efflux from root cells prevents accumulation of toxic levels of Na⁺ in the cytosol and transport of Na⁺ to the shoot. Molecular genetic analysis in *Arabidopsis sos* mutants have led to the identification of a plasma membrane Na⁺/H⁺ antiporter, SOS1, which plays a crucial role in sodium extrusion from root epidermal cells under salinity. The *SOS1* transcript level is up-regulated under salt stress. The *sos1* mutant plants show

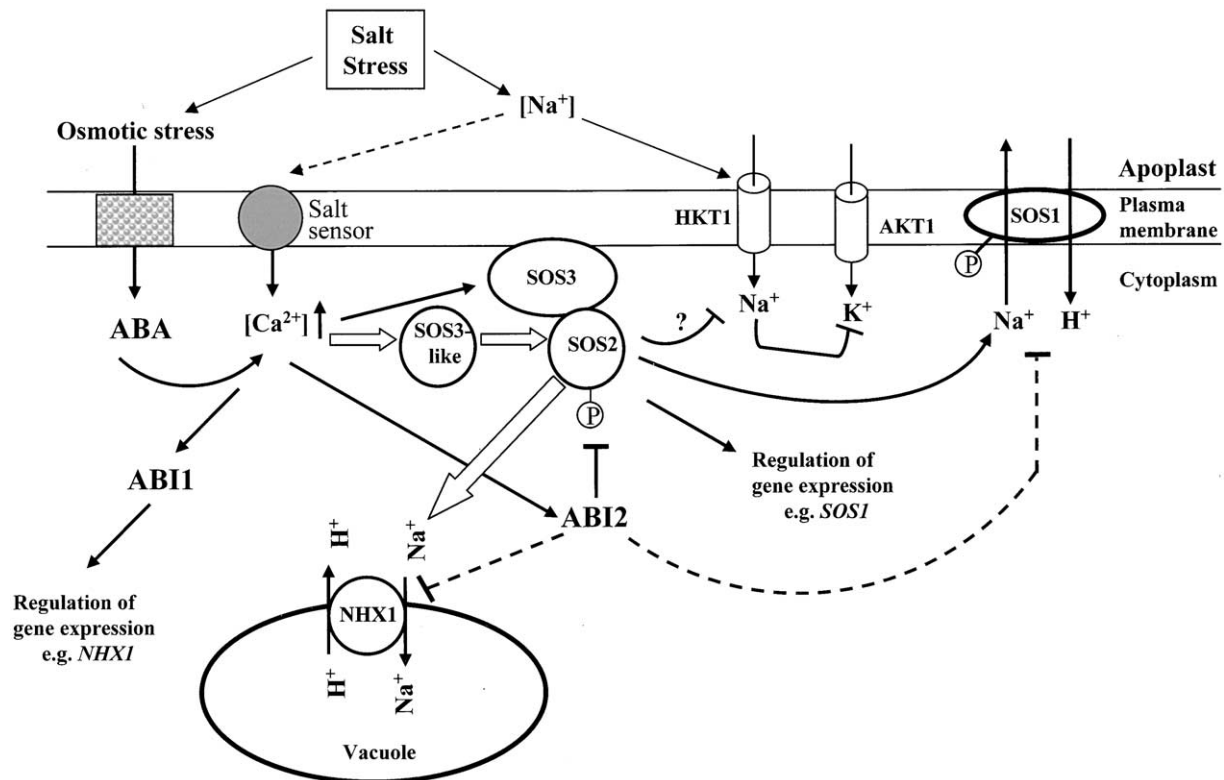


Fig. 1. SOS signaling pathway for ion homeostasis under salt stress in *Arabidopsis*. Salt stress elicited Ca^{2+} signals are perceived by SOS3, which activates the protein kinase SOS2. Activated SOS2 phosphorylates SOS1, a plasma membrane Na^+/H^+ antiporter, which then transports Na^+ out of the cytosol. The transcript level of SOS1 is regulated by the SOS3-SOS2 kinase complex. SOS2 also activates the tonoplast Na^+/H^+ antiporter that sequesters Na^+ into the vacuole. Na^+ entry into the cytosol through the Na^+ transporter HKT1 may also be restricted by SOS2. ABI1 regulates the gene expression of NHX1, while ABI2 interacts with SOS2 and negatively regulates ion homeostasis either by inhibiting SOS2 kinase activity or the activities of SOS2 targets. Double arrow indicates SOS3-independent and SOS2-dependent pathway.

hypersensitivity to salt stress (100 mM NaCl), and accumulate more Na^+ in shoots than wild-type plants. Sodium efflux by SOS1 is also vital for salt tolerance of meristem cells such as growing root-tips and shoot apex as these cells do not have large vacuoles for sodium compartmentation (Shi et al., 2000 & 2002). Isolated plasma membrane vesicles from *sos1* mutants show significantly less inherent as well as salt stress-induced Na^+/H^+ antiporter activity than vesicles from wild-type plants (Qiu et al., 2002). The expression of *SOS1* is ubiquitous, but stronger in epidermal cells surrounding the root-tip, as well as parenchyma cells bordering the xylem. Thus, SOS1 functions as a Na^+/H^+ antiporter on the plasma membrane and plays a crucial role in sodium efflux from root cells and the long distance Na^+ transport from root to shoot (Shi et al., 2002). Indeed, transgenic *Arabidopsis* plants overexpressing *SOS1* have lower Na^+ in the xylem transpirational stream and in shoots compared with wild-type plants. These plants also show enhanced salt tolerance, measured in terms of their growth, ability to bolt and flower at increasing concentrations of salt stress (50–200 mM NaCl); while, control plants became necrotic and have failed to bolt (Shi et al., 2003).

Sodium efflux through SOS1 under salinity is regulated by SOS3–SOS2 kinase complex (Fig. 1). In *Arabidopsis*, salt-stress induced calcium signatures are sensed by SOS3, a Ca^{2+} sensor protein with three calcium bind-

ing EF hands and an N-myristoylation motif (Liu and Zhu, 1998; Ishitani et al., 2000). Mutations that disrupt either calcium binding (*sos3-1*) or myristoylation (G2A) of SOS3 cause salt-stress hypersensitivity in *Arabidopsis* plants (Ishitani et al., 2000). The SOS3 gene product transduces a salt stress-elicited calcium signal by activating SOS2, a ser/thr protein kinase with an N-terminal kinase catalytic domain that is similar to that of yeast SNF1 and animal AMP-activated kinase, and a unique C-terminal regulatory domain. The C-terminal regulatory domain of SOS2 consists of an autoinhibitory FISL motif (Liu et al., 2000), deletion of which results in constitutive activation of SOS2 (Guo et al., 2001). Under salt stress, SOS3 binds to the FISL motif of SOS2 and activates its substrate phosphorylation (protein kinase) activity (Halfter et al., 2000). Activated SOS2 then phosphorylates SOS1, and results in activation of antiporter activity of SOS1. The Na^+/H^+ exchange activity of isolated plasma membranes vesicles from *sos3* and *sos2* mutants is significantly less than that of wild-type plants. Consistent with this finding, these mutants also accumulate higher levels of Na^+ , similar to those accumulated by the *sos1* mutant (Quintero et al., 2002). Overexpression of an active form of SOS2 could overcome the salt hypersensitivity of *sos2* and *sos3* mutants and enhanced the salt tolerance of transgenic *Arabidopsis* (Guo et al., 2004). The *SOS1* up-regulation under salt stress is also impaired in *sos2* and *sos3* mutants. Hence, the SOS3–

SOS2 signaling pathway positively regulate salt-stress induced *SOS1* gene expression and/or transcript stability as well as *SOS1* transporter activity (Shi et al., 2003).

In addition to increasing cytosolic calcium, salt-stress induced ABA accumulation also appears to regulate the SOS pathway through the ABA insensitive 2 (ABI2) protein phosphatase 2C. ABI2 interacts with the protein phosphatase interaction (PPI) motif of SOS2. This interaction is abolished by the *abi2-1* mutation, which enhances tolerance of seedlings to salt shock (150 mM NaCl) and causes ABA insensitivity. Hence, the wild-type ABI2 may negatively regulate salt tolerance either by inactivating SOS2, or the SOS2 regulated Na⁺/H⁺ antiporters such as SOS1 or NHX1 (Fig. 1; Ohta et al., 2003).

Sodium Compartmentation

A positive turgor is indispensable for expansion growth of cells and stomatal openings in plants. A decrease in water potential due to soil salinity causes osmotic stress that leads to turgor loss. Plants have evolved an osmotic adjustment (active solute accumulation) mechanism that maintains water uptake and turgor under osmotic stress conditions. For osmotic adjustment, plants use inorganic ions such as Na⁺ and K⁺ and/or synthesize organic compatible solutes such as proline, betaine, polyols, and soluble sugars. Vacuolar sequestration of Na⁺ is an important and cost-effective strategy for osmotic adjustment that also reduces the Na⁺ concentration in the cytosol. Na⁺ sequestration into the vacuole depends on expression and activity of Na⁺/H⁺ antiporters as well as on V-type H⁺-ATPase and H⁺-PPase. These phosphatases generate the necessary proton gradient required for activity of Na⁺/H⁺ antiporters.

Overexpression of *AVP1*, a vacuolar H⁺-pyrophosphatase in *Arabidopsis* enhanced sequestration of Na⁺ into the vacuole and maintained higher relative water content in leaves. These plants also show higher salt- and drought-stress tolerance than that of wild type (Gaxiola et al., 2001). The tonoplast Na⁺/H⁺ antiporter *NHX1* gene is induced by both salinity and ABA in *Arabidopsis* (Shi and Zhu, 2002) and rice (Fukuda et al., 1999). The *AtNHX1* promoter contains putative ABA responsive elements (ABRE) between -736 and -728 from the initiation codon. *AtNHX1* expression under salt stress is partially dependent on ABA biosynthesis and ABA signaling through ABI1. Salt-stress induced up-regulation of *AtNHX1* expression is lower in ABA deficient mutants (*aba2-1* and *aba3-1*) and in the ABA insensitive mutant, *abi1-1* (Shi and Zhu, 2002). Comparing tonoplast Na⁺/H⁺-exchange activity (mainly due to *AtNHX1*) between wild type and mutants (*sos1*, *sos2*, and *sos3*) shows that SOS2 also regulates the tonoplast exchange. The impaired tonoplast Na⁺/H⁺-exchange activity in vitro from isolated *sos2* tonoplasts could be restored to levels in wild type by adding activated SOS2 protein. Since the tonoplast Na⁺/H⁺-exchange activity is not affected in the *sos3* mutant, the tonoplast Na⁺/H⁺-exchange activity is not regulated by SOS3. SOS2 has been found to interact with plant calcium sensor

proteins such as SOS3, SCABP1 (SOS3-like calcium-binding proteins 1), SCABP3, SCABP5, and SCABP6. One of these SCABPs may signal SOS2 to regulate the tonoplast Na⁺/H⁺-exchange activity (Fig. 1; Qiu et al., 2003).

Transgenic *Arabidopsis* plants overexpressing *AtNHX1* have showed significantly higher salt (200 mM NaCl) tolerance than wild-type plants (Apse et al., 1999). Since tomato is a highly salt-sensitive crop (Table 1), an effort has been made to improve its salt tolerance by overexpressing *AtNHX1*. These tomato transgenics grow and produce fruits in the presence of very high salt concentrations (200 mM NaCl). Yield and fruit quality of transgenic tomato plants under salinity are equivalent to those of control plants grown under nonstress conditions (Zhang and Blumwald, 2001). Similar results have been reported for transgenic canola (*Brassica napus* L.) overexpressing *AtNHX1* (Zhang et al., 2001).

Compatible Osmolytes

Although use of ions for osmotic adjustment may be energetically more favorable than biosynthesis of organic osmolyte under osmotic stresses, many plants accumulate organic osmolytes to tolerate osmotic stresses. These osmolytes include proline, betaine, polyols, sugar alcohols, and soluble sugars. Glycine betaine and trehalose act as osmoprotectants by stabilizing quaternary structures of proteins and highly ordered states of membranes. Mannitol serves as a free-radical scavenger. Proline serves as a storage sink for carbon and nitrogen and a free-radical scavenger. It also stabilizes subcellular structures (membranes and proteins), and buffers cellular redox potential under stress. Hence, these organic osmolytes are known as osmoprotectants (Bohnert and Jensen, 1996; Chen and Murata, 2000). Genes involved in osmoprotectant biosynthesis are up-regulated under salt stress, and concentrations of accumulated osmoprotectants correlate with osmotic stress tolerance (Zhu, 2002). Analysis of the *Arabidopsis t365* mutant supports the involvement of osmoprotectants in salt tolerance. The *t365* mutant is impaired in the *S*-adenosyl-L-methionine phosphoethanolamine *N*-methyltransferase (*PEAMT*) gene. The *PEAMT* enzyme catalyzes conversion of phosphoethanolamine to phosphocholine, which is a precursor of glycinebetaine biosynthesis (Mou et al., 2002).

Salt tolerance of transgenic tobacco engineered to over-accumulate mannitol was first demonstrated by Tarczynski et al. (1993). Genetically engineered over-production of compatible osmolytes in transgenic plants such as *Arabidopsis*, rice, wheat, and *Brassica* has also been shown to enhance stress tolerance as measured by germination, seedling growth, survival, recovery, photosystem II yield, and seed production under very high salt and osmotic stresses. The observed salt tolerance was attributed to the osmoprotectant effect of compatible osmolytes rather than their contribution to osmotic adjustment (Table 2). It is interesting to note that glycine betaine- (Kishitani et al., 2000) and trehalose- (Garg et al., 2002) overproducing transgenic rice plants accumulated fewer Na⁺ ions, and maintained K⁺ uptake,

Table 2. Salt-stress tolerance of transgenic plants over-producing compatible osmolytes.

Gene and source	Transgenic plants	Stress tolerant traits	Reference
<u>Mannitol</u>			
<i>E. coli mt1D</i> (mannitol-1-phosphate dehydrogenase)	tobacco	fresh weight, plant height and flowering under salinity stress	Tarczynski et al., 1993
<i>E. coli mt1D</i>	<i>Arabidopsis</i>	germination at 400 mM NaCl	Thomas et al., 1995
<i>E. coli mt1D</i>	tobacco	salt-stress tolerance; mannitol contributed only to 30-40% of the osmotic adjustment	Karakas et al., 1997
<i>E. coli mt1D</i>	wheat (<i>Triticum aestivum</i> L.)	only 8% biomass reduction when compared to 56% reduction in control plants in 150 mM NaCl stress	Abebe et al., 2003
<u>D-Ononitol</u>			
<i>IMT1</i> (myo-inositol <i>O</i> -methyl transferase) of common ice plant	tobacco	drought and salinity stress	Sheveleva et al., 1997
<u>Sorbitol</u>			
<i>Stpd1</i> (sorbitol-6-phosphate dehydrogenase) of apple, driven by CaMV 35S promoter	Japanese persimmon	tolerance in Fv/Fm ratio under NaCl stress	Gao et al., 2001
<u>Glycine betaine</u>			
<i>Arthrobacter globiformis Coda</i> (choline oxidase)	<i>Arabidopsis</i>	germination at 300 mM NaCl; seedling growth at 200 mM NaCl; retention of PSII activity at 400 mM NaCl	Hayashi et al., 1997
<i>A. globiformis Coda</i> targeted to the chloroplasts or cytosol	rice	faster recovery after 150 mM NaCl stress	Sakamoto et al., 1998; Mohanty et al., 2002
<i>A. globiformis Coda</i>	<i>Brassica juncea</i> (L.) Czernj.	germination in 100–150 mM NaCl; seedling growth in 200 mM NaCl	Prasad et al., 2000
<i>E. coli</i> choline dehydrogenase (<i>betA</i>) and betaine aldehyde dehydrogenase (<i>betB</i>) genes	tobacco	biomass production of greenhouse grown plants under salt stress; faster recovery from photo inhibition under high light, salt stress and cold stresses	Holmstrom et al., 2000
<i>Atriplex hortensis</i> betaine aldehyde dehydrogenase (<i>BADH</i>) gene under maize ubiquitin promoter	wheat (<i>Triticum aestivum</i> L.)	seedling growth in 0.7% (=120 mM) NaCl	Guo et al., 2000
Barley peroxisomal <i>BADH</i> gene	rice	stability in chlorophyll fluorescence under 100 mM NaCl stress; accumulates less Na ⁺ and Cl ⁻ ions but maintained K ⁺ uptake	Kishitani et al., 2000
<u>Proline</u>			
<i>Vigna aconitifolia</i> L. <i>P5CS</i> (Δ^1 -pyrroline-5-carboxylate synthetase) gene	tobacco	root growth; flower development	Kishor et al., 1995
<i>Vigna aconitifolia</i> L. <i>P5CS</i> gene under barley HVA22 promoter	rice	faster recovery after a short period of salt stress	Zhu et al., 1998
Mutated gene of <i>Vigna aconitifolia</i> L. <i>P5CS</i> which encode <i>P5CS</i> enzyme that lacks end product (proline) inhibition	tobacco	improved seedlings tolerance and low free radical levels at 200 mM NaCl	Hong et al., 2000
Antisense proline dehydrogenase gene	<i>Arabidopsis</i>	tolerant to high salinity (600 mM NaCl); constitutive freezing tolerance (-7°C)	Nanjo et al., 1999
<u>Trehalose</u>			
<i>E. coli otsA</i> (Trehalose-6-phosphate synthase) and <i>otsB</i> (Trehalose-6-phosphate phosphatase) bi-functional fusion gene (<i>TPSP</i>) under the control of ABA responsive promoter or Rubisco small subunit (<i>rbcS</i>) promoter	rice	root and shoot growth at 4 wk of 100 mM NaCl stress; survival under prolonged salt stress; maintenance of high K ⁺ /Na ⁺ ratio; Low Na ⁺ accumulation in the shoot; maintained high PSII activity and soluble sugar levels	Garg et al., 2002
<i>E. coli TPSP</i> under maize ubiquitin promoter	rice	better seedling growth and PSII yield under salt, drought and cold stresses	Jang et al., 2003

Thus, these plants retained optimal K⁺/Na⁺ ratios necessary for cellular functions. Whether ion homeostasis in these transgenics was either due to direct regulation of ion transporters or to maintenance of cellular integrity by protecting membranes and proteins from oxidative damage was not known and needs to be determined.

Although enhanced synthesis and accumulation of compatible solutes under osmotic stresses are well documented, little is known about the signaling cascades that regulate the compatible solute biosynthesis in higher plants. A signaling cascade similar to that of the yeast Mitogen Activated Protein Kinase-High Osmotic Glycerol 1 (MAPK-HOG1) pathway may regulate osmolyte biosynthesis (Zhu, 2002). A putative osmosensory two-

component hybrid histidine kinase, *ATHK1*, from *Arabidopsis* is implicated in osmosensing under salt stress based on induced expression and ability to complement the yeast double mutant lacking both osmosensors (*sln1Δ sho1Δ*). By analogy to SLN1 of yeast, the *Arabidopsis* ATHK1 is also probably active at low osmolarity. Active ATHK1 may inactivate a response regulator by phosphorylation. Inactivation of ATHK1 under high osmolarity may result in the accumulation of nonphosphorylated active form of the response regulator, which then stimulates osmolyte biosynthesis in plants by activating a MAPK pathway(s) (Urao et al., 1999). Transcriptome analyses also show induction of receptor-like kinase genes in *Arabidopsis* under salt stress (Kreps

et al., 2002; Seki et al., 2002). However, genetic and molecular evidences to support the role of these proteins in osmotic stress sensing and compatible osmolyte biosynthesis are lacking.

ABA may also regulate osmolyte biosynthesis in plants under salt stress. Osmotic stress-induced ABA accumulation has been shown to regulate the *P5CS* gene involved in proline biosynthesis (Xiong et al., 2001a). Proline induces the expression of salt-stress responsive genes, which have proline responsive elements (*PRE*, *ACTCAT*) in their promoters (Satoh et al., 2002; Oono et al., 2003). Better understanding of the salt-stress signaling pathway that regulates compatible osmolyte biosynthesis will help to devise better breeding and genetic engineering strategies.

LEA-Type Proteins

Osmotic stresses induce late-embryogenesis-abundant (LEA) proteins in vegetative tissues, which impart dehydration tolerance to vegetative tissues of plants. These LEA-type proteins are encoded by RD (responsive to dehydration), ERD (early responsive to dehydration), KIN (cold inducible), COR (cold regulated), and RAB (responsive to ABA) genes in different plant species (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). Accumulation levels of these proteins correlate with stress tolerance in various plant species suggesting protective roles under osmotic stress. Transgenic rice plants engineered to overexpress a barley *LEA* gene, *HVA1*, under control of the rice actin 1 promoter exhibit better stress tolerance under 200 mM NaCl and drought stress than wild-type plants (Xu et al., 1996). Expression of LEA-type genes under osmotic stresses is regulated

by both ABA-dependent and independent signaling pathways (Fig. 2). Promoters of LEA-like genes contain dehydration responsive elements/C-Repeat (DRE/CRT), ABA-responsive elements (ABREs), and/or MYB/MYC recognition elements. The DRE/CRT elements regulate gene expression in response to dehydration (salt, drought, and cold stresses); while, ABRE and MYB/MYC elements control gene expression in response to ABA under abiotic stresses (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000).

Genetic analysis of ABA-deficient *Arabidopsis* mutants, *los5* and *los6*, has revealed that ABA is necessary for the salt-stress induced expression of some *Arabidopsis* *LEA* genes (Xiong et al., 2001a; Xiong et al., 2002). Ca^{2+} and/or H_2O_2 act as second messengers of ABA induced stomatal closure and gene expression under abiotic stresses (Leung and Giraudat, 1998; Schroeder et al., 2001). Transient expression analysis has revealed that IP_3 and cADPR-gated calcium channels are involved in ABA induced Ca^{2+} concentration changes, and these Ca^{2+} transients regulate expression of LEA-type genes, such as *RD29A* and *KIN2* (Wu et al., 1997). Genetic evidence from the *fry1* (*fiery 1*) mutant, defective in inositol polyphosphate 1-phosphatase, has demonstrated that IP_3 metabolism is critical for ABA and abiotic stress signaling (Xiong et al., 2001b). Salt-stress/ABA induced Ca^{2+} signals are at least partially transduced through calcium-dependent protein kinases (CDPKs). Transient expression analyses in maize protoplasts have shown that an increase in cytosolic Ca^{2+} concentration activates CDPKs, which in turn induce the stress responsive *HVA1* promoter. Moreover, expression of CDPKs is under the negative control of ABI1 protein phospho-

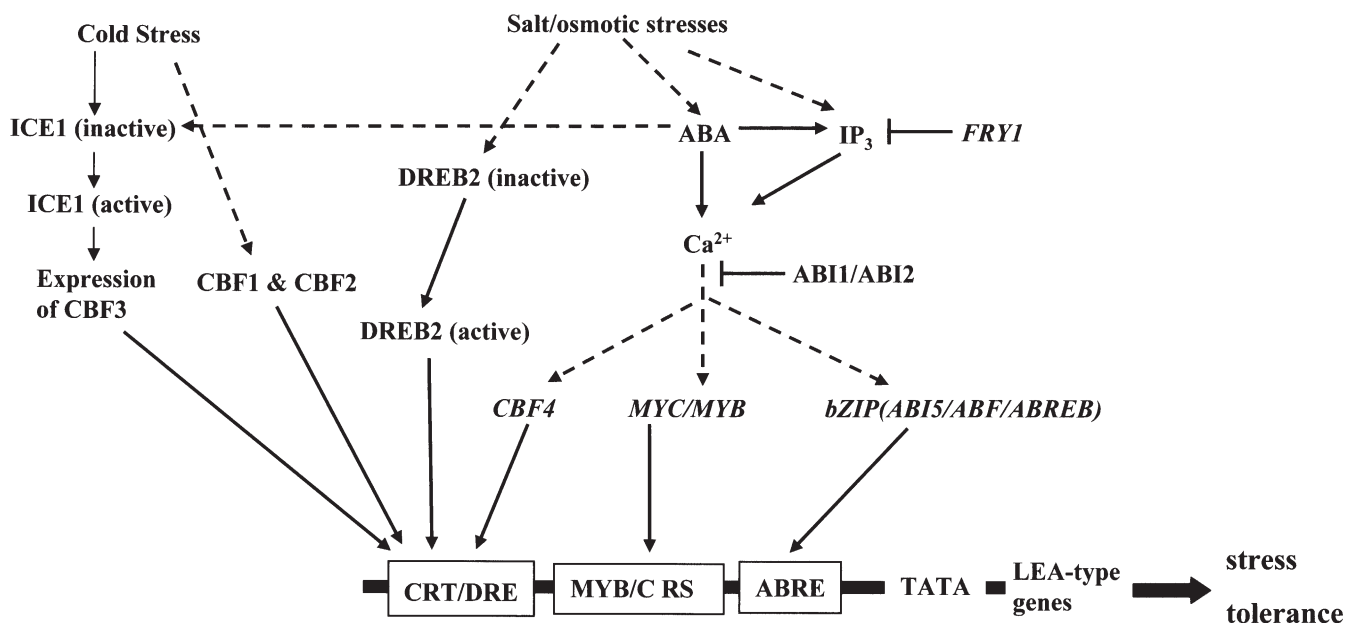


Fig. 2. LEA-type gene transcription under abiotic stresses in *Arabidopsis*. ABA-independent DREB2 and ABA-dependent CBF4 transcription factors transactivate DRE/CRT cis-elements in the promoters of LEA type genes. ABA-dependent pathways regulate LEA type genes through MYC/MYB and bZIP type transcription factors. ABA-dependent signaling is mediated through IP_3 and Ca^{2+} . *FRY1* negatively regulates IP_3 levels. ABA induced Ca^{2+} signaling is negatively regulated by ABI1/2 protein phosphatase 2C. Low temperature stress activates ICE1 a myc-like bHLH transcription factor, which binds to myc type cis-elements of CBF3 promoter and induces CBF3 expression. CBFs bind to the CRT/DRE cis-elements on the promoter of LEA-type genes and induce expression of these genes.

tase 2C (Sheen, 1996). Overexpressing *OsCDPK7* in transgenic rice enhances induction of a LEA-type gene (*RAB16A*) and salt/drought tolerance; while, transgenic suppression of *OsCDPK7* causes hypersensitivity to salt/drought stress (Saijo et al., 2000). Signaling components regulating CDPK-activated gene expression are yet to be defined.

ABA-dependent expression of LEA-type genes under osmotic stress is regulated by basic Leucine-Zipper and MYB/MYC type transcription factors that recognize ABRE (Uno et al., 2000) and MYB/MYC recognition sequences (Abe et al., 2003), respectively (Fig. 2). *Arabidopsis* bZIP transcription factors, *ABREB1* (ABA-responsive element binding protein 1 = *ABF2*) and *ABREB2* (= *ABF4*) genes, are up-regulated by drought, NaCl, and ABA. The induction of the *RD29B* promoter-*GUS* by *ABREB1* and *ABREB2* in *Arabidopsis* leaf protoplasts under osmotic stress is repressed in *aba2* and *abil* mutants but is enhanced in an *era1* mutant. ABA is necessary for the activation of *ABREB1* and *ABREB2* (Uno et al., 2000). Constitutive overexpression of *ABF3* and *ABREB2* (= *ABF4*) in *Arabidopsis* enhance expression levels of target LEA-type genes (*RAB18* and *RD29B*). These transgenic plants are hypersensitive to ABA, sugar, and salt stress during germination but are drought tolerant at the seedling stage (Kang et al., 2002).

Salt stress-inducible basic-helix-loop-helix type transcription factors as well as *AtMYC2* (= *RD22BP1*) and *AtMYB2* regulate ABA-responsive gene expression in *Arabidopsis*. Constitutive overexpression of *AtMYC2* and *AtMYB2* results in constitutive expression of *RD22* and *AtADH*, and expression levels are further increased following ABA treatment. These transgenic plants are hypersensitive to ABA during germination. In contrast, *atmyc2* mutation decreases *RD22* and *AtADH* expression. Transgenic *Arabidopsis* plants overexpressing *AtMYC2* and *AtMYB2* show higher osmotic stress tolerance as measured by electrolyte leakage from cells (Abe et al., 2003), although their salt-stress tolerance is not known. ABA signaling via ABFs and MYC/MYB and their targets must be investigated to better understand sensitivity during germination and tolerance during the vegetative growth in transgenic plants.

ABA-independent regulation of LEA-type genes is mediated by transcription factors that activate DRE/CRT *cis*-elements of LEA-type protein encoding genes. These transcription factors are called either C-repeat Binding Proteins (*CBFs*) or Dehydration Responsive Element Binding Proteins (*DREBs*). *Arabidopsis* *DREBs* are classified into two classes, *DREB1* (*DREB1A* = *CBF3*, *DREB1B* = *CBF1*, and *DREB1C* = *CBF2*), and *DREB2* (*DREB2A* and *DREB2B*). Expression of *CBF1*, *CBF2*, and *CBF3* is induced by low temperature stress; while, expression of *DREB2A* and *DREB2B* is induced by dehydration and salt stresses (Liu et al., 1998; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Fig. 2). Recently, a *DREB1* homolog of *Arabidopsis* *CBF4* has been cloned. *CBF4* shows ABA-dependent expression under drought stress (Haake et al., 2002). Overexpression of *CBF* (*CBF1-4*) genes has resulted in acti-

vation of *DRE/CRT cis* elements leading to expression of LEA-type genes (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Jaglo et al., 2001; Haake et al., 2002). The CBF pathway for expression of LEA-type genes is conserved across plant species such as *Arabidopsis*, wheat, *Brassica napus* (Jaglo et al., 2001), tomato (Hsieh et al., 2002a, 2002b), barley, and rice (Dubouzet et al., 2003). Similar to *Arabidopsis* *DREB2*, rice *OsDREB2A* is also induced by dehydration and salt stress (Dubouzet et al., 2003). Recently, we have identified ICE1 (Inducer of CBF Expression 1), a MYC-type basic helix-loop-helix transcription factor, as an upstream regulator of these DREB/CBF transcription factors under cold stress (Chinnusamy et al., 2003). Upstream transcription factors that regulate the expression of DREB2/CBF4 transcription factors under osmotic stresses (salt or dehydration) have yet to be identified.

Constitutive overexpression of *CBFs* strongly activated expression of several LEA-type genes, enhancing freezing and osmotic stress tolerance of transgenic *Arabidopsis* (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999) and *Brassica napus* (Jaglo et al., 2001), and chilling and drought tolerance of tomato (Hsieh et al., 2002a, 2002b). However, constitutive overexpression of *CBFs* resulted in severe growth retardation and reduction in seed production, even under a normal environment (Liu et al., 1998). Transgenic *Arabidopsis* overexpressing *CBF3* under the transcriptional control of the stress responsive *RD29A* promoter showed no growth retardation when compared to control plants. These transgenic *Arabidopsis* plants showed constitutive low-levels of expression of *LEA* genes and enhanced expression under cold, dehydration, and salt stresses. Both the *RD29A::CBF3* and *CaMV35S::CBF3* transgenic plants showed enhanced tolerance to freezing, drought, and salt stresses, but tolerance levels of *RD29A::CBF3* transgenics were significantly higher than those of *CaMV35S::CBF3* transgenic plants. Recovery and survival of seedlings after soaking in 600 mM NaCl solution for 2 h was 79 and 18% for *RD29A::CBF3* transgenic and control plants, respectively (Kasuga et al., 1999). Transgenic wheat plants expressing *RD29A::CBF3* also showed enhanced osmotic stress tolerance (Pellegrineschi et al., 2002). In addition to enhanced expression of LEA-type genes, multiple abiotic stress tolerance of *CBF*-overexpressing transgenic plants might also be in part due to accumulation of compatible osmolytes (Gilmour et al., 2000) and enhanced oxidative stress tolerance (Hsieh et al., 2002a, 2002b). It was not clear how osmolyte biosynthesis and antioxidant defense pathways were activated in *CBF*-overexpressing plants. Genome-wide expression analysis showed that *CBF* overexpression also induced transcription factors such as AP2 domain proteins (*RAP2.1* and *RAP2.6*), a putative zinc finger protein and R2R3-MYB73 (Fowler and Thomashow, 2002), that may regulate osmolyte biosynthesis and antioxidant defense genes. Hence, genetic engineering of *CBFs* and potentially other transcription factors under stress specific promoters in crops appears to be a viable approach for engineering tolerance to multiple stresses, including salt stress.

Tobacco-stress-induced-gene 1 (*Tsi1*) encoding a DNA-binding protein with an EREBP/AP2 DNA binding motif is rapidly induced by salt stress but not by drought or ABA. Overexpression of *TSII* in tobacco enhanced retention of chlorophyll content when leaves were floated on 400 mM NaCl solution for 48 or 72 h (Park et al., 2001), although the targets of TSI1 are not known.

Oxidative Stress Management

Abiotic stresses including salt-stress induce accumulation of ROS that are detrimental to cells at high concentrations because they cause oxidative damage to membrane lipids, proteins, and nucleic acids (Smirnov, 1993; Gomez et al., 1999; Hernandez et al., 2001). Plants employ antioxidants (e.g., ascorbate, glutathione, α -tocopherol, and carotenoids) and detoxifying enzymes, such as superoxide dismutase, catalase, and enzymes of ascorbate-glutathione cycle to combat oxidative stress. The activity and expression levels of the genes encoding detoxifying enzymes are probably enhanced by ROS under abiotic stresses. Transgenic plants overexpressing ROS scavenging enzymes, such as superoxide dismutase (reviewed by Alscher et al., 2002), ascorbate peroxidase (Wang et al., 1999), and glutathione *S*-transferase/glutathione peroxidase (Roxas et al., 1997, 2000) showed increased tolerance to osmotic, temperature, and oxidative stresses. Overexpression of the tobacco *NtGST/GPX* gene (encoding a bifunctional enzyme with both glutathione *S*-transferase and glutathione peroxidase activity) in transgenic tobacco plants has improved salt- and chilling-stress tolerance because of enhanced ROS scavenging and prevention of membrane damage (Roxas et al., 1997, 2000).

The *Arabidopsis pst1* (*photoautotrophic salt tolerance I*) mutant is more tolerant to salt stress than the wild type. The observed salt tolerance was attributed to higher activities of superoxide dismutase and ascorbate peroxidase than those in wild-type *Arabidopsis* (Tsu-gane et al., 1999). Thus, ROS detoxification is an important part of salt-stress tolerance.

Salt stress (Gomez et al., 1999; Hernandez et al., 2001) and ABA (Guan et al., 2000; Pei et al., 2000) induce enhanced production of H₂O₂, which may also act as a second messenger at sublethal concentrations to regulate antioxidant defense genes under abiotic stresses. ABA-dependent ROS production is catalyzed by NADPH oxidase as revealed with analysis of the *atrbohD/F* double mutant of *Arabidopsis*, which is impaired in ABA-induced ROS production (Kwak et al., 2003). ABA-elicited H₂O₂ production is negatively regulated by the ABI2 protein in guard cells (Murata et al., 2001).

Potential sensors of ROS may include redox sensitive receptors-like kinases and two component histidine kinases that likely activate a mitogen-activated protein kinase (MAPK) module. Salt stress triggers activation and enhanced gene expression of a MAPK signaling cascade, some components of which are common for both salt and ROS (for review, Chinnusamy and Zhu, 2003). Salt stress rapidly (within 5–10 min) activates *Arabidopsis* mitogen activated protein kinase kinase kinase (AT-

MEKK1; Ichimura et al., 1998), mitogen activated protein kinase kinase (AtMKK2; Teige et al., 2004), and MAPKs (ATMPK3, ATMPK4 and ATMPK6; Mizoguchi et al., 1996; Ichimura et al., 2000). Activated AtMEKK1 has been shown to activate AtMPK4 and AtMPK6 in vitro and in vivo (Huang et al., 2000; Teige et al., 2004). The MAPK phosphatase 1 (*mkp1*) mutant of *Arabidopsis* is more resistant to salinity stress. A yeast two-hybrid screen showed that MKP1 could interact with AtMPK4 (Ulm et al., 2002). Thus, the salt-stress regulated MAPK cascade consisting of AtMEKK1, AtMEK1/AtMKK2, and AtMPK4 is negatively regulated by MKP1.

Salt-stress induced ROS signaling in *Arabidopsis* may also be transduced by ANP1 (a MAPKKK), AtMPK3, and AtMPK6 along with its positive regulator Nucleoside Diphosphate Kinase 2 (AtNDPK2) (Kovtun et al., 2000; Moon et al., 2003). Transgenic tobacco plants overexpressing a constitutively active tobacco *NPK1* (ortholog of ANP1) show enhanced tolerance to salinity and other abiotic stresses (Kovtun et al., 2000). AtNDPK2 interacts with and activates both ATMPK3 and ATMPK6. Transgenic *Arabidopsis* overexpressing *AtNDPK2* accumulate lower levels of ROS and show enhanced tolerance to salinity and other abiotic stresses (Moon et al., 2003). In rice, gene expression as well as kinase activity of rice MAPK (*OsMAPK5*) is regulated by ABA, by biotic, and abiotic stresses including salt, drought, wounding, and cold (Xiong and Yang, 2003). Thus, diverse abiotic stress signals converge at MAPK cascades that appear to regulate antioxidant defense under abiotic stresses in plants.

Transgenic overexpression of *NPK1* in tobacco (Kovtun et al., 2000), *NDPK2* in *Arabidopsis* (Moon et al., 2003), and *OsMAPK5* in rice (Xiong and Yang, 2003), increased tolerance to several abiotic stresses, including salt stress, probably by enhancing ROS detoxification. Constitutively active NPK1 activated a MAPK cascade that activates promoters of stress-responsive genes, such as Glutathione-*S*-transferase (*GST6*) and *HSP18.2* but not *RD29A* (Kovtun et al., 2000). Promoters of genes encoding ROS detoxifying enzymes contain antioxidant responsive elements (ARE), ABA responsive elements (ABRE), heat shock elements (HSE), and redox-regulated transcription factors: nuclear factor kappa-B (NFkB) and the activator protein-1 (AP-1) recognition cis-elements (Vranová et al., 2002). However, transacting factors and their regulators need to be identified.

Genetic Engineering of Salt-Tolerant Crops

Transgenic approaches by manipulation of ion homeostasis, osmoprotectant accumulation, LEA-type proteins, and ROS scavenging capacity have demonstrated the capabilities of engineering salt-tolerant crops. Although abiotic stress tolerance is known to be governed by multiple genes, significant increases in salt tolerance can be achieved by single gene manipulations as revealed by *SOS1*- (Shi et al., 2003) and *NHX1*- (Apse et al., 1999; Zhang and Blumwald, 2001; Zhang et al., 2001) overexpressing transgenic plants. These transgenics are capable of growing and producing flowers at salt concentra-

tions of up to 200 mM NaCl (20 dS/m), which is lethal to wild-type plants. Most crop plants are susceptible to this concentration of salinity (Table 1). In addition, these transgenics do not exhibit any obvious growth abnormalities or changes in the quality of the consumable product, similar to results with *NHX1* overexpressing transgenic tomato and *Brassica* plants. Hence genetic engineering for ion homeostasis by tissue specific overexpression of *SOS1*, *NHX1*, and their positive regulator, the active form of *SOS2*, will help in significant improvement in salt tolerance.

Transgenic analysis of osmolyte over-production has shown that osmoprotectants can protect plants against short term and high intensity salt stress (Table 2), but stress tolerance must be evaluated for the entire life period of plants. Polyol over-accumulating transgenic plants show growth abnormalities, including sterility (Karakas et al., 1997; Sheveleva et al., 1998). Further, compartmentation of these osmoprotectants may also be required for enhanced tolerance. For example, rice transgenic plants overexpressing choline oxidase targeted to chloroplasts show better tolerance to photo-inhibition under salt- and low-temperature stresses than plants overexpressing choline oxidase targeted to the cytosol (Sakamoto et al., 1998). Hence, the level of expression of transgene, substrate requirement, metabolic flux, and cellular compartmentation of osmoprotectants should be considered for engineering osmoprotectant accumulation. Overexpression of antioxidant systems has been shown to protect transgenic plants from abiotic stresses; however, in some cases, transgenic plants did not show enhanced stress tolerance. Pyramiding of chloroplastic and mitochondrial Mn-superoxide dismutases in alfalfa (*Medicago sativa* L.) resulted in lower biomass production as compared with the transgenic plants expressing either one of the Mn-superoxide dismutases (Samis et al., 2002). Engineering for antioxidant systems may alter the pool size of ROS, involved in developmental and stress signaling, and hence their possible effects warrant careful examination.

Overexpression of signaling components and transcription factors lead to expression of their target transcriptome, which consists of multiple genes contributing to stress adaptation. Overexpression of CBF transcription factors from constitutive or stress-inducible promoters has been shown to confer enhanced tolerance in the seedling stage to multiple abiotic stresses. However, constitutive overexpression has led to growth abnormalities. Hence, overexpression of CBF transcription factors/transcriptome engineering under stress-inducible promoters is preferable for genetic engineering of multiple abiotic stress tolerance.

Some developmental phases of plants are more sensitive to salt stress than others. For example, in rice, seedling growth, seedling survival, and fertility are all adversely affected at salinity levels (EC_e) higher than 1.9, 3.4, and 4.5 dS m^{-1} , respectively (Zeng and Shannon, 2000). Hence, it is imperative to understand the tissue and developmental specificity of salt-stress tolerance. Quantitative trait loci (QTL) can be considered as a cluster of related genes that may be under the transcriptional

control of a regulatory gene. Transgenic manipulation of a single regulatory gene may be sufficient to regulate a gene cluster. Genetic and transgenic analyses have clearly demonstrated that manipulation of upstream transcription factor or signaling genes can lead to the activation of multiple target tolerance effector genes, and thus significantly improves abiotic stress tolerance.

Conclusions and Prospects

Only a few facets of myriad salt stress-tolerant traits found in nature have been unraveled today by application of molecular tools such as gene disruption and transgenic approaches. The SOS pathway regulates ion homeostasis under salt stress. An unknown salt-stress sensor induces cytosolic calcium signals, which are transduced by the SOS3-SOS2 kinase complex. Activated SOS2 kinase regulates sodium efflux and sequestration of sodium into the vacuole by activating Na^+/H^+ antiporters of plasma membrane and tonoplast, respectively. Osmotic homeostasis and stress damage control appear to be regulated by salt stress-induced ABA, ROS, a putative osmosensory histidine kinase (AtHK1), and MAPK cascades. However, these signaling pathways are not yet understood in terms of their components and targets. It appears possible to engineer salt-tolerant crops by manipulating Na^+/H^+ antiporters (plasma membrane and tonoplast) and the CBF transcriptome in the near future. Exploitation of other signaling components, osmolyte over-production, and antioxidant defense requires further consideration. In the future, pyramiding regulatory genes controlling various aspects of salt tolerance (i.e., ionic and osmotic homeostasis, and damage control) in a single transgenic plant is expected to yield very high levels of tolerance to salt and other related stresses. Most of the transgenic plants discussed here are model plants, and stress tolerance has been evaluated under controlled growing conditions for short durations. As the rate of transpiration is one of the major determinants of the concentration of salt accumulation in shoots, salt tolerance must be evaluated in the field conditions. The effects of stresses in relation to plant ontogeny should be assessed at realistic stress levels and under various combinations that naturally occur in the field.

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