SALT STRESS SIGNALING AND MECHANISMS OF PLANT SALT TOLERANCE

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INTRODUCTION

Soil salinity of agricultural land has led to the breakdown of ancient civilizations. Even today it threatens agricultural productivity in 77 mha of agricultural land, of which 45 mha (20% of irrigated area) is irrigated and 32 mha (2.1% of dry land) is unirrigated (1). Salinization is further spreading in irrigated land because of improper management of irrigation and drainage. Rain, cyclones and wind also add NaCl into the coastal agricultural land. Soil salinity often leads to the development of other problems in soils such as soil sodicity and alkalinity. Soil sodicity is the result of the binding of Na^+ to the negatively charged clay particles, which leads to clay swelling and dispersal. Hydrolysis of the Na-clay complex results in soil alkalinity. Thus, soil salinity is a major factor limiting sustainable agriculture.

The USDA salinity laboratory defines saline soil as having electrical conductivity of the saturated paste extract (EC_e) of 4 dS m^{-1} (1 dS m^{-1} is approximately equal to 10 mM NaCl) or more. High concentrations of soluble salts such as chlorides of sodium, calcium and magnesium contribute to the high electrical conductivity of saline soils. NaCl contributes to most of the soluble salts in saline soil.

The development of salinity-tolerant crops is the need of the hour to sustain agricultural production. Conventional breeding programs aimed at improving crop tolerance to salinity have limited success because of the complexity of the trait (2). Slow progress in breeding for salt-tolerant crops can be attributed to the poor understanding of the molecular mechanisms of salt tolerance. Understanding the molecular basis of plant salt tolerance will also help improve drought and extreme-temperature-stress tolerance, since osmotic and oxidative stresses are common to these abiotic stresses. The salt-

tolerant mechanisms of plants can be broadly described as ion homeostasis, osmotic homeostasis, stress damage control and repair, and growth regulation (3). This chapter reviews recent progress in understanding salt-stress signaling and breeding/genetic engineering for salt -tolerant crops.

EFFECT OF SALINITY ON PLANT DEVELOPMENT

Salinity affects almost all aspects of plant development, including germination, vegetative growth and reproductive development. Soil salinity imposes ion toxicity, osmotic stress, nutrient (N, Ca, K, P, Fe, Zn) deficiency and oxidative stress on plants. Salinity also indirectly limits plant productivity through its adverse effects on the growth of beneficial and symbiotic microbes. High salt concentrations in soil impose osmotic stress and thus limit water uptake from soil. Sodium accumulation in cell walls can rapidly lead to osmotic stress and cell death (1). Ion toxicity is the result of replacement of K^+ by Na^+ in biochemical reactions, and Na^+ and Cl^- -induced conformational changes in proteins. For several enzymes, K^+ acts as cofactor and cannot be substituted by Na^+. High K^+ concentration is also required for binding tRNA to ribosomes and thus protein synthesis (3, 4). Ion toxicity and osmotic stress cause metabolic imbalance, which in turn leads to oxidative stress (5).

In terms of plant tolerance to salinity, plants are classified as halophytes, which can grow and reproduce under high salinity (>400mM NaCl), and glycophytes, which cannot survive high salinity. Most of the grain crops and vegetables are natrophobic (glycophytes) and are highly susceptible to soil salinity, even when the soil EC_e is < 4 dS m^{-1}. Crops such as bean (*Phaseolus vulgaris*), eggplant (*Solanum melongena*), corn (*Zea mays*), potato (*Solanum tuberosum*), and sugarcane (*Saccharum officinarum*) are highly susceptible, with a threshold EC_e of <2 dS m^{-1}, whereas sugar beet (*Beta vulgaris*) and barley (*Hordeum vulgare*) can tolerate an EC_e of up to 7 dS m^{-1}. Sugar beets and barley are highly sensitive to salinity during germination but are highly tolerant during the later phases of crop development (6; http://www.ussl.ars.usda.gov/saltoler.htm). Soil type (particularly Ca^{2+} and clay content), rate of transpiration (which determines the amount of salt transported to the shoot for any given rate of salt uptake and loading to the xylem by roots) and radiation may further alter the salt tolerance of crops.

Salinity affects photosynthesis mainly through a reduction in leaf area, chlorophyll content and stomatal conductance, and to a lesser extent through a decrease in photosystem II efficiency (7). The adverse effects of salinity on plant development are more profound during the reproductive phase. Figure 1 shows the adverse effect of salinity on vegetative and reproductive development and the differential sensitivity of yield components to different intensities of salt stress in rice (8). Wheat plants stressed at 100 to 175 mM NaCl showed a significant reduction in spikelets per spike, delayed spike emergence and reduced fertility, which results in poor grain yield. However, Na^+ and Cl^- concentrations in the shoot apex of these wheat plants were below 50 and 30 mM, respectively, which is too low to limit metabolic reactions (9). Hence, the adverse effects of salinity may be attributed to the salt-stress effect on the cell cycle and differentiation.

Salinity arrests the cell cycle transiently by reducing the expression and activity of cyclins and cyclin-dependent kinases that results in fewer cells in the meristem, thus limiting growth (10). In *Arabidopsis*, the reduction in root meristem size and root growth during salt stress is correlated with the downregulation of *CDC2a* (cyclin-dependent kinase), *CycA2;1* and *CycB1;1* (mitotic cyclins) (11). The activity of cyclin-dependent kinase is diminished also by post-translational inhibition during salt stress (10). Salt stress-induced abscisic acid (ABA) may also mediate cell cycle regulation. ABA upregulates the expression of the inhibitor of cyclin-dependent kinase *ICK1*, which is a negative regulator of CDC2a (12). Salinity adversely affects reproductive development by inhibiting microsporogenesis and stamen filament elongation, enhancing programmed cell death in some tissue types, ovule abortion and senescence of fertilized embryos. In *Arabidopsis*, 200 mM NaCl stress causes as high as 90% ovule abortion (13).

GeneChip microarray transcriptome analysis of salt-stressed (100 mM NaCl stress for 3 hours) *Arabidopsis* plants revealed approximately 424 and 128 genes were upregulated (>2 fold) in roots and leaves, respectively (14). In *Arabidopsis*, cDNA microarray analysis showed that about 194 genes were upregulated and about 89 were downregulated by salt stress (15). In rice, of 1700 cDNAs analysed, approximately 57 genes were upregulated by NaCl stress (16). Many of the NaCl upregulated genes were also upregulated by dehydration, cold and ABA (14-16), which suggests that some of the stress responses are common to all these abiotic stresses. These results show that plant

responses to salt stress are controlled by several genes and salt tolerance is a complex phenomenon.

PERCEPTION OF SALT STRESS

The ability of the plant to combat environmental stress is determined by the efficiency of the plant to sense the environmental stress and activate its defense machinery. Salt stress is perceived by plants as ionic and osmotic stresses. Excess Na^+- and Cl^--induced conformational changes in protein structure and membrane depolarization can lead to the perception of ion toxicity. Plasma membrane proteins, ion transporters and/or Na^+-sensitive enzymes have been hypothesized as sensors of toxic Na^+ concentrations in extracellular and intracellular sites. Many transporters with long cytoplasmic tails similar to that of SOS1 (*S*alt *O*verly *S*ensitive 1, the plasma membrane Na^+/H^+ antiporter) have been implicated as being sensors of the molecule transported by that transporter. Similar to the sugar permease BglF in *E. coli* and the ammonium transporter Mep2p in yeast, SOS1 has been proposed to be one of the potential sensors of Na^+ ions in plants (17). Another potential candidate sensor is a Na^+-K^+ co-transporter of *Eucalyptus camaldulensis*. This transporter showed increased ion uptake under hypo-osmotic conditions when expressed in *Xenopus laevis* oocytes (18).

Salinity-imposed osmotic stress leads to cell turgor loss and cell volume change. Hence, the potential sensors of osmotic stress include membrane-associated stretch-activated channels, cytoskeleton (microtubules and microfilaments), and transmembrane protein kinases, such as two-component histidine kinases. One of the putative sensors of osmotic stress in *Arabidopsis* is the hybrid two-component histidine kinase *ATHK1* (19). The proposed role of AtHK1 in salt tolerance is discussed later in this chapter.

SECOND MESSENGERS

The ameliorative effects of Ca^{2+} in maintaining plant growth under salinity (20) and Ca^{2+}-induced ion channel discrimination against Na^+ (21) have been well known for a long time. In addition to its effect on preventing Na^+ entry into cells, Ca^{2+} acts as a signaling molecule in salt stress signaling (22, 23). Cytosolic Ca^{2+} oscillations during salt stress are regulated through the activities of mechanosensitive and ligand-gated Ca^{2+}

channels on the plasma membrane, endoplasmic reticulum and vacuole (3, 17). Excess Na^+-induced membrane depolarization may activate mechanosensitive Ca^{2+} channels to generate Ca^{2+} signature under salt stress (4, 17). Pharmacological studies and genetic analysis have shown the involvement of inositol 1,4,5-tris phosphate (IP_3)-gated Ca^{2+} channels in the regulation of Ca^{2+} signature during salt stress (24-26). The *FRY1* locus of *Arabidopsis* encodes an inositol polyphosphate 1-phosphatase, which catabolizes IP_3. The *Arabidopsis fry1* mutant is impaired in inositol polyphosphate 1-phosphatase and thus exhibits impaired ABA-induced IP_3 transients. The *fry1* mutation leads to sustained accumulation of IP_3 and hypersensitivity to ABA, cold and salt stress. Thus, IP_3 plays a crucial role in cytosolic Ca^{2+} oscillations during ABA, salt and cold stress signaling (26). Salinity stress also leads to synthesis of the plant stress hormone ABA (27-29) and accumulation of reactive oxygen species (ROS) (5). Calcium and/or H_2O_2 act as second messengers of ABA-induced stomatal closure and gene expression under abiotic stresses (30, 31). Transient expression analysis revealed that IP_3 and cyclic ADP ribose (cADPR)-gated calcium channels are involved in ABA-induced cytosolic Ca^{2+} oscillations (32). ABA induces the expression and activity of ADP-ribosyl (ADPR) cyclase, which synthesizes cADPR (33). Involvement of a heterotrimeric GTP-binding (G)-protein has been demonstrated in ABA signal transduction during guard cell regulation (34). Since ABA synthesis is induced under salinity, the G-protein-associated receptors may also elicit Ca^{2+} signatures during salinity stress. Salt stress-induced Ca^{2+} signatures are then sensed and transduced by calcium sensor proteins, namely SOS3 and SOS3-like calcium binding proteins (SCaBPs), calcium-dependent protein kinases (CDPKs), and calmodulins (CaMs).

ION HOMEOSTASIS

Plants achieve ion homeostasis by restricting the uptake of toxic ions, maintaining the uptake of essential ions and compartmentalization of toxic ions into the vacuole of specific tissue types. In most crop plants, Na^+ is the primary cause of ion toxicity, and hence, management of cellular Na^+ concentration is critical for salt tolerance (4). Sodium ions can be kept below the toxic level in the cytosol by i) restricting Na^+ entry at the root cortex cells, ii) excreting Na^+ from root cells into soil, iii) retrieving Na^+ from the

transpirational xylem stream to recirculate it to the roots, iv) storing Na^+ in the vacuole of mature cells, and v) excreting Na^+ through salt glands (3). Among these mechanisms, Na^+ excretion through salt glands is important only in halophytes. Biochemical, electrophysiological and molecular genetic evidence show that the SOS pathway plays a crucial role in the regulation of cellular and whole plant ion homeostasis (Fig. 2) (17).

SODIUM UPTAKE

Restricting Na^+ entry into the root cells and then into the transpirational stream is critical to prevent a buildup of toxic levels of salt in the shoot. Both glycophytes and halophytes must exclude about 97% of the Na^+ present in the soil at the root surface to prevent toxic levels of Na^+ accumulation in the shoots (35). Sodium entry into the transpirational stream depends upon the amount of Na^+ uptake by Na^+ and nonspecific cation transporters and the proportion of water entry in the apoplastic/bypass pathway into the xylem. Na^+ from the soil gains the initial entry into the cells of the root epidermis and cortex. The casparian strip in the endodermis plays a crucial role in preventing apoplastic Na^+ influx into the root stele. Compared with *Arabidopsis*, halophytes such as salt cress (*Thellungiella halophila*) develop both an extra endodermis and a cortex cell layer in roots (36). In maize seedlings stressed at 200 mM NaCl, the casparian strip radial width was increased by 47% compared with control seedlings (37). This feature may help to reduce the Na^+ entry into the transpirational stream. In crops such as rice, water entry into the xylem through the bypass pathway accounts for all the Na^+ buildup in the shoots, whereas in crops such as wheat, transport protein-mediated Na^+ uptake accounts for most of the Na^+ buildup in the shoots (38). Silica deposition and polymerization of silicate in the endodermis and rhizodermis blocks Na^+ influx through the apoplastic pathway in the roots of rice (39). Regulation of these anatomical and morphological changes in root development during salt stress needs further understanding.

Sodium uptake is mediated by both voltage-dependent and -independent cation channels. The role of voltage-independent cation channels in Na^+ uptake is poorly understood. Voltage-dependent cation channels such as K^+ inward rectifiers (HKT, HAK and KUP) mediate Na^+ uptake into root cells. Sodium competes with K^+ uptake through Na^+-K^+ cotransporters and may also block the K^+-specific transporters of root cells (17).

Expression studies in yeast cells revealed that high-affinity K^+-uptake activity of both *Arabidopsis* AtKUP1 and barley HvHAK1 is inhibited by millimolar concentrations of Na^+ (40, 41). Cellular K^+ concentration can be maintained by the activity/expression of inward-rectifying K^+-specific transporters under high salinity. Salt stress, as well as K^+ starvation, upregulates the expression of *Mesembryanthemum crystallinum* high-affinity K^+ transporter genes (*McHAK*s). McHAKs specifically mediate K^+ uptake and show high discrimination for Na^+ at high salinity (42). In contrast, high-affinity K^+ transporters (HKTs) of wheat (43, 44), *Arabidopsis* (45) and *Eucalyptus* (18) act as low-affinity Na^+ transporters when expressed in *Xenopus* oocytes. HKT transporters of *Eucalyptus camaldulensis* and wheat possess Na^+:K^+ symport activity but mediate mainly Na^+ transport under high salinity (18, 43). The expression of *OsHKT1* is significantly downregulated in salt-tolerant rice cv. Pokkali as compared with salt-sensitive rice cv. IR29 during 150 mM NaCl stress (46). Transgenic wheat plants expressing antisense wheat *HKT1* showed significantly less $^{22}Na^+$ uptake and enhanced growth under high salinity as compared with control plants (47). This evidence suggests that HKT1 homologs contribute to Na^+ influx during salt stress and downregulation of *HKT1* may help limit Na^+ influx to roots.

In yeast, *HAL1* and *HAL3* regulate the expression of P-type ATPase, Na^+ efflux and K^+ uptake. Transgenic overexpression of the yeast *HAL1* gene enhanced salt tolerance of melon shoots *in vitro* (48), tomato (49, 50) and watermelons (51). Transgenic tomato plants overexpressing yeast *HAL1* showed increased K^+ accumulation. Irrigation with 35 mM NaCl to plants till maturity decreased the control plant fruit yield by 57.5%, whereas transgenic plants showed 24-42% decreased fruit yield. However, under normal growing conditions, the transgenic lines were less productive than the wild type (50). Overexpression of the *Arabidopsis HAL3a* gene also enhanced the salt tolerance of transgenic *Arabidopsis* (52).

Electrophysiological evidence suggests that cyclic nucleotides (cAMP and cGMP) may minimize Na^+ influx into the cell by downregulating voltage-independent cation channels in *Arabidopsis* (53). Exposure of *Arabidopsis* plants to salt and osmotic stress results in increased cytosolic cGMP concentration within 5 seconds (54). Pyrridoxal-5-phosphate is a cofactor for transaminases involved in the biosynthesis of aminoacids that

are precursors for nucleotide biosynthesis. The *Arabidopsis sos4* mutant defective in a pyridoxal kinase gene showed hypersensitive root growth under NaCl and KCl stress and accumulated more Na^+ but less K^+ than the wild type. Pyridoxal-5-phosphate and its derivatives act as ligands for P2X receptor ion channels in animals (55). Pyridoxal-5-phosphate may regulate Na^+ efflux by SOS1, because SOS1 contains a putative pyridoxal-5-phosphate binding domain (3). Thus, regulation of K^+ and Na^+ uptake by pyridoxal-5-phosphate and cyclic nucleotides may help in plant salt tolerance. Signaling pathways(s) that regulate Na^+ and K^+ uptake by higher plants during salinity need further study.

Comment [G1]: Your phrase "….and more Na but less K" is highly obscure. What are you really trying to say?

SODIUM EFFLUX

Sodium efflux from root cells is a frontline defense that prevents the accumulation of toxic levels of Na^+ in the cytosol and Na^+ transport to the shoot. Plasma membrane Na^+/H^+ antiporters pump out Na^+ from root cells. In *Arabidopsis*, the plasma membrane Na^+/H^+ antiporter SOS1 mediates Na^+ efflux, and its activity is regulated by the SOS3-SOS2 kinase complex during salt stress (Fig. 2) (17). Salt stress-induced Ca^{2+} signatures are sensed by SOS3. SOS3 has 3 calcium-binding EF hands and an N-myristoylation motif and shows sequence similarity to the calcineurin B subunit of yeast and neuronal Ca^{2+} sensors of animals (56, 57). Calcineurin is a protein phosphatase (PP2B) that regulates salt tolerance in yeast. SOS3 and SOS3-like calcium-binding proteins (SCaBPs) identified in *Arabidopsis* differ from yeast calcineurin structurally and functionally. SCaBPs do not have a calcineurin A subunit catalytic domain. Unlike calcineurin, which activates protein phosphatases, SOS3 activates the Ser/Thr protein kinase during salt stress. Thus, SOS3 and SCaBPs are a new class of Ca^{2+} sensor proteins in higher plants. Mutations that disrupt either Ca^{2+} binding (*sos3-1*) or myristoylation (G2A) of SOS3 cause salt stress hypersensitivity in *Arabidopsis* (57). SOS3 binds Ca^{2+} with low affinity as compared with other Ca^{2+}-binding proteins such as caltractin and calmodulin (57). The differences in the affinity of these Ca^{2+} sensors may be employed by cells to distinguish various Ca^{2+} signals. SOS3 transduces the salt stress signal by activating SOS2, a Ser/Thr protein kinase with an N-terminal kinase catalytic domain that is similar to that of yeast sucrose nonfermenting 1 (SNF1) and animal AMP-activated kinase (AMPK), and has a

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unique C-terminal regulatory domain. The C-terminal regulatory domain of SOS2 consists of an autoinhibitory FISL motif (58). Under normal cellular conditions, the catalytic and regulatory domains of SOS2 interact with each other, likely preventing substrate phosphorylation by blocking substrate access. Yeast two-hybrid and *in vitro* binding assay have shown that in the presence of Ca^{2+}, SOS3 binds to and activates the SOS2 kinase (59). The FISL motif in the regulatory domain of SOS2 is necessary and sufficient for interacting with SOS3, and deletion of this FISL motif constitutively activates SOS2. Replacing Thr^{168} in the kinase domain by Asp also results in a constitutively active SOS2 kinase (60).

Molecular genetic analyses led to the identification of targets of the SOS3-SOS2 regulatory pathway. One of the targets of the SOS pathway is SOS1. SOS1 has significant protein sequence homology and conserved domains similar to that of the plasma membrane Na^+/H^+ antiporter from bacteria, fungi and animals. The expression of *SOS1* is ubiquitous but stronger in epidermal cells surrounding the root tip and in parenchyma cells bordering the xylem. The expression of the SOS1::GFP fusion protein and anti-SOS1 antibody confirmed that SOS1 is localized in the plasma membrane of root and leaf cells (61-63). *sos1* mutant plants show hypersensitivity to salt stress (100 mM NaCl) and accumulate more Na^+ in shoots than do wild-type plants (61). Isolated plasma membrane vesicles from *sos1* mutants showed significantly less inherent as well as salt stress-induced Na^+/H^+ antiporter activity than did vesicles from the wild type (64). This evidence shows that SOS1 functions as a Na^+/H^+ antiporter on the plasma membrane and plays a crucial role in sodium efflux from the root cells. Indeed, transgenic *Arabidopsis* plants overexpressing *SOS1* exhibited lower levels of Na^+ in the xylem transpirational stream and in the shoot than wild-type plants and enhanced salt tolerance. Transgenic plants grew, bolted and flowered with increasing concentrations of salt stress (50 to 200 mM NaCl), whereas control plants become necrotic and did not bolt (65). The expression level of *SOS1* is also significantly higher in salt cress (*T. halophila*) than in *Arabidopsis*, even in the absence of salt stress (66).

The Na^+/H^+ exchange activity of SOS1 is regulated by the SOS3-SOS2 complex under salt stress. Isolated plasma membrane vesicles from *sos3* and *sos2* mutants showed significantly less Na^+/H^+ exchange activity 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. However, the addition of activated SOS2 is sufficient to rescue the Na^+/H^+ exchange activity of plasma membrane vesicles from *sos3* and *sos2* mutants (64, 67). The SOS3-SOS2 kinase complex phosphorylates the SOS1 protein and activates SOS1 Na^+/H^+ antiporter activity (67). *SOS1* upregulation during salt stress is also under the regulatory control of the SOS pathway, as shown by the impaired expression of *SOS1* in salt-stressed *sos2* and *sos3* mutants (60). Overexpression of the active form (Thr168 to Asp mutation) of *SOS2* under the control of the CaMV 35S promoter (*35S::T/DSOS2*) rescued the *sos2* and *sos3* mutants under salinity conditions. Transgenic *Arabidopsis* expressing *35S::T/DSOS2* showed enhanced SOS1 transporter activity and better vegetative and reproductive growth than wild-type plants when grown in soil irrigated with 200 mM NaCl (68). Co-expression of *SOS1*, *SOS2* and *SOS3* rescued yeast cells deficient in Na^+ exchangers. Co-expression of *SOS2* and *SOS3* significantly increased SOS1-dependent Na^+ tolerance in the yeast mutant (67). This evidence demonstrates that SOS3 senses the salt-stress induced Ca^{2+} signals and activates SOS2 kinase, which in turn regulates the Na^+/H^+ exchange activity and expression of *SOS1* (Fig. 2) (17).

SODIUM COMPARTMENTATION

Soil salinity decreases soil water potential, which leads to osmotic stress. To maintain water uptake during osmotic stress, plants have evolved a mechanism known as osmotic adjustment. Osmotic adjustment is active accumulation of solutes such as inorganic ions (Na^+ and K^+) and organic solutes (proline, betaine, polyols and soluble sugars). Vacuolar sequestration of Na^+ is an important and cost-effective strategy for osmotic adjustment and at the same time reduces the cytosolic Na^+ concentration during salinity. Vacuolar Na^+/H^+ antiporters use the proton gradient generated by vacuolar H^+-adenosine triphosphatase (H^+-ATPase) and H^+-inorganic pyrophosphatase (H^+-PPase) for Na^+ sequestration into the vacuole. Hence, coordinated regulation of the Na^+/H^+ antiporters, H^+-ATPase and H^+-PPase is crucial for salt tolerance. Salt stress induces tonoplast H^+-ATPase and H^+-PPase activities (69). Transgenic *Arabidopsis* plants overexpressing *AVP1* (H^+-PPase) showed enhanced sequestration of Na^+ into the vacuole

and maintained higher relative leaf water content and enhanced salt and drought stress tolerance as compared with the wild type (70). NO-mediated signaling is implicated in the activation of plasma membrane H^+-ATPase (71), but the regulators of tonoplast H^+-ATPases and H^+-PPase are yet to be identified.

Vacuolar Na^+ sequestration is further regulated at the level of expression and activity of tonoplast Na^+/H^+ antiporters (NHXs). Expression of *NHX1* is induced by salinity and ABA in *Arabidopsis* (72, 73), rice (74) and cotton (75). The expression level of *NHX1* is correlated with genotypic differences in salt tolerance in cotton (75). Complementation studies showed that *AtNHX1* (72) and *OsNHX1* (69) could complement the yeast *nhx1* mutant. Transgenic *Arabidopsis* plants overexpressing *AtNHX1* showed significantly higher salt (200 mM NaCl) tolerance than wild-type plants (76). Transgenic tomato plants overexpressing *AtNHX1* were able to grow and produce fruits in the presence of very high salt concentrations (200 mM NaCl) at which wild-type plants did not survive. The yield and fruit quality of transgenic tomato plants under salt stress were equivalent to that of control plants under non-stress conditions (77). Similar results were obtained with transgenic canola (*B. napus*) overexpressing *AtNHX1* (78). These tomato and canola plants accumulated high concentrations of Na^+ in older leaves but not in reproductive parts (77, 78). Inspired by these results, transgenic rice plants overexpressing *Atriplex gmelini NHX1* (79), rice overexpressing *OsNHX1* (69) and tobacco overexpressing *Gossipium hirsutum NHX1* (75) were engineered. These transgenic plants showed better salt tolerance than control plants in the vegetative stage. However, transgenic rice plants overexpressing *OsNHX1* did not show a K^+-to-Na^+ ratio significantly different from that of control plants (69). Analysis of salt tolerance of the *osnhx1* null mutant of rice (69) may shed further light on the role of NHX1 in salt tolerance.

The SOS pathway and ABA regulate *AtNHX1* gene expression and its antiporter activity under salt stress. The promoter of *AtNHX1* contains putative ABA responsive elements (ABRE) between –736 to –728 from the initiation codon. *AtNHX1* expression under salt stress depends in part on ABA biosynthesis and ABA signaling through ABI1, because the salt stress-induced upregulation was reduced in ABA-deficient mutants (*aba2-1* and *aba3-1*) and the ABA-insensitive mutant, *abi1-1* (73). In *G. hirsutum*,

GhHNX1 expression is induced by ABA and appears to be regulated by MYB/MYC-type transcription factors (75). Analysis of the tonoplast Na^+/H^+-exchange activity in wild type and *sos* mutants (*sos1*, *sos2* and *sos3*) revealed that SOS2 also regulates tonoplast Na^+/H^+-exchange activity. The impaired tonoplast Na^+/H^+-exchange activity from isolated *sos2* tonoplasts could be restored to the wild-type level by the addition of activated SOS2 protein. Since the Na^+/H^+-exchange activity is unaffected in the *sos3* mutant, regulation of tonoplast Na^+/H^+-exchange activity by SOS2 is independent of SOS3 (80). SOS2 has been found to interact with plant calcium sensor proteins such as SOS3, SCaBP1, SCaBP3, SCaBP5 and SCaBP6 (81). One of these SCaBPs may signal SOS2 to regulate tonoplast Na^+/H^+-exchange activity (Fig 2) (80). SOS2 also has an additional SOS3-independent role in regulating the vacuolar H^+/Ca^{2+} antiporter VCX1, which plays a crucial role in regulating the duration and amplitude of cytosolic Ca^{2+} oscillations (82).

SODIUM TRANSPORT FROM SHOOTS TO ROOTS

Many of the glycophytes have limited ability to sequester Na^+ in leaf vacuoles. Therefore, these plants recirculate excess Na^+ from the leaf to the root. Sodium transport from shoots to roots is probably mediated by SOS1 and HKT1 in *Arabidopsis*. Under salt stress (100 mM NaCl), Na^+ accumulation in shoots of *sos1* mutant plants was greater than that of the wild type. Strong expression of *SOS1* in cells bordering the xylem suggests that SOS1 mediates either Na^+ release into or Na^+ retrieval from the xylem stream, depending on salt stress intensity, and thus is critical for controlling long-distance Na^+ transport from roots to shoots (62). Comparison of the expression pattern of *HKT1* in wheat and *Arabidopsis* revealed that AtHKT and wheat HKT1 might have different functions. *AtHKT1* is mainly expressed in phloem tissues but not in root peripheral cells, whereas wheat HKT1 is localized to the root epidermis and leaf vasculature. The sodium overaccumulation in shoots *2-1* (*sas2-1*) mutant of *Arabidopsis* showed significantly higher shoot Na^+ content but lower root Na^+ content and Na^+ concentration in the phloem sap exuding from leaves than the wild type. *sas2-1* mutation impaired *AtHKT1* and thus its Na^+ transport activity in *Xenopus* oocytes (83). T-DNA mutation in the *AtHKT1* gene has resulted in higher shoot Na^+ content and lower root Na^+ content than that in the wild type (84). Moreover AtHKT1 does not show significant K^+ transport activity in *Xenopus*

oocytes. A single-point mutation, Ser-68 to glycine, was sufficient to restore K^+ permeability to AtHKT1 (84). These results show that AtHKT1 probably mediates Na^+ loading into the phloem sap in shoots and unloading in roots and thus helps to maintain a low Na^+ concentration in shoots (83). The *Arabidopsis athkt1*Δ mutation suppresses the salt hypersensitivity and K^+-deficient phenotype of *sos3* (85). Hence, the SOS pathway may regulate and coordinate the activities of AtHKT1 and SOS1 to control Na^+ transport from shoots to roots.

Salt stress-induced ABA accumulation, in addition to cytosolic Ca^{2+}, may also regulate the SOS pathway through the ABI2 protein phosphatase 2C. ABI2 interacts with the protein phosphatase interaction motif of SOS2. This interaction is abolished by the *abi2-1* mutation, which enhances the tolerance of seedlings to salt shock (150 mM NaCl) and causes ABA insensitivity. Hence the wild-type ABI2 may negatively regulate salt tolerance by inactivating SOS2 or the SOS2-regulated ion channels such as HKT1, Na^+/H^+ antiporters, SOS1 and NHX1 (Fig 2; 86).

Transgenic manipulations of ion homeostasis have demonstrated the possibilities of genetic engineering salt-tolerant crop plants. Although multiple genes govern salt-stress tolerance, significant increases in salt tolerance has been achieved by single-gene manipulations, as revealed by *SOS1*- (65) and *NHX1*- (76, 77, 78) overexpressing transgenics. These transgenics were able to grow and flower at a salt concentration of 200 mM NaCl (~20 dS m^{-1}), which is lethal to wild-type plants. Most crop plants are susceptible to this concentration of salinity (6). In addition, these transgenics do not produce any obvious growth abnormalities or change in the quality of the consumable product, as shown by *NHX1*-overexpressed transgenic tomato and *Brassica* (77, 78). Hence, genetic engineering for ion homeostasis by tissue-specific overexpression of *SOS1*, *NHX1* and their positive regulator, the active form of SOS2, will help improve the salt tolerance of crop plants.

STRESS DAMAGE PREVENTION AND REPAIR

Stress damage prevention and repair pathways are necessary for cell survival at metabolically inhibitory levels of ionic or osmotic stresses. These strategies may include osmotic adjustment, osmoprotectant accumulation, oxidative stress management,

induction of stress proteins (LEA-type proteins, chaperonin, etc.) and other physiological adaptations such as modifications in root and shoot growth and transpiration.

OSMOPROTECTANTS

Plants accumulate organic osmolytes such as proline, betaine, polyols, sugar alcohols and soluble sugars to tolerate osmotic stress. These organic solutes protect plants from abiotic stress by i) osmotic adjustment, which helps in turgor maintenance; ii) detoxification of reactive oxygen species; and iii) stabilization of the quaternary structure of proteins (87). Polyols and proline act as antioxidants (88). Proline also stabilizes subcellular structures (membranes and proteins) and buffers cellular redox potential under stress. Glycine betaine and trehalose stabilize the quaternary structures of proteins and highly ordered state of membranes. Glycine betaine also reduces lipid peroxidation during salinity stress. Hence, these organic osmolytes are known as osmoprotectants (3, 87-89). In addition to these organic osmoprotectants, polyamines also play a significant role in salt-stress tolerance. Mutations that impair arginine decarboxylase (ADC catalyzes the first committed step in polyamine biosynthesis) results in salt hypersensitivity (90, 91). Genes involved in osmoprotectant biosynthesis are upregulated under salt stress, and the concentrations of accumulated osmoprotectants correlates with osmotic stress tolerance (3, 89). Halophytes such as *T. halophila* accumulate significantly higher concentrations of proline than *Arabidopsis*, even under nonstress conditions (66). Genetic analysis of the *Arabidopsis t365* mutant impaired in the S-adenosyl-L-methionine:phosphoethanolamine N-methyltransferase (*PEAMT*) gene involved in glycine betaine biosynthesis (Fig. 3) showed hypersensitivity to salt stress (92). Thus, glycine betaine accumulation is critical for salt tolerance. Several efforts have been made to engineer salt and other abiotic stress resistance in plants through genetic manipulation of osmoprotectant metabolism in plant. The pathways of various osmoprotectant biosynthesis are shown in Fig. 3. Genes of these pathways that are employed in genetic engineering for salt tolerance are briefly reviewed in Table 1.

Table 1. Metabolic engineering of osmoprotectant accumulation for salt-stress tolerance in plants

Gene	Plant	Stress tolerance	Reference
Glycine Betaine			
Arthrobacter globiformis choline oxidase (*CodA*)	*Arabidopsis*	Germination in 300 mM NaCl; Seedling growth in 200 mM NaCl; Retention of PSII activity at 400 mM NaCl	93
A. globiformis CodA under CaMV 35S promoter	*Arabidopsis*	Exposure of 40-day-old plants to 100 mM NaCl stress for 3 days resulted in flower bud abortion and a decrease in number of seeds per silique in control plants. These adverse effects were less in transgenic plants	94
A. globiformis CodA	Rice	Transgenic plants in which *CodA* is targeted to the chloroplasts were more tolerant to photo inhibition under 150 mM NaCl salt stress and cold stress than *CodA* expression in cytosol.	95
A. globiformis CodA	*Brassica juncea*	Better germination in 100-150 mM NaCl and seedling growth in 200 mM NaCl	96
Arthrobacter pascens choline oxidase (*COX*)	Arabidopsis, *B. napus*, and tobacco	No significant differences in osmotic potential between transgenic and non-transgenic plants.	97
E. coli choline dehydrogenase (*betA*) and betaine aldehyde dehydrogenase (*betB*) genes	Tobacco	Biomass production of greenhouse grown transgenic plants was greater than that of wild-type plants under salt stress; Faster recovery from photo inhibition under high light, salt stress and cold stress	98
Atriplex hortensis BADH driven by maize ubiquitin promoter	*Triticum aestivum*	Seedling growth in 0.7 % NaCl	99
Peroxisomal *BADH* of barley	Rice	Stability in chlorophyll fluorescence; accumulation of fewer Na^+ and Cl^- ions and more K^+ ions in shoots under 100 mM NaCl stress	100
Proline			
Vigna aconitifolia L. P5CS (Δ^1-pyrroline-5-carboxylate synthetase) gene	Tobacco	Better root growth and flower development under salt stress	101
V. aconitifolia L. P5CS that lacks end product	Tobacco	Improved seedling tolerance and low free radical levels at 200 mM NaCl	102

(proline) inhibition			
V. aconitifolia L. *P5CS* gene under barley HVA22 promoter	Rice	Faster recovery after a short period of salt stress to seedlings	103
Antisense proline dehydrogenase gene	*Arabidopsis*	Tolerant to high salinity (600 mM NaCl); constitutive freezing tolerance (-7°C) at vegetative stage	104
Antisense Δ^1-pyrroline-5-carboxylate reductase gene under heat stress inducible promoter	Soybean	Antisense transgenic plants (vegetative stage) accumulate less proline and failed to survive 6 days of drought at 37°C, whereas control plants survived	105
Trehalose			
E. coli OstA (Trehalose 6P synthase) & *OstB* (Trehalose 6P Phosphatase) driven by ABA responsive promoter	Rice	Higher survival rate and K^+/Na^+ ratio, low Na^+ accumulation in the shoot, high PSII activity, high root & shoot growth under 100mM NaCl stress at vegetative stage	106
E. coli OstA & *OstB* driven by maize ubiquitin promoter	Rice	Enhanced seedling growth and PSII yield under salt, drought and cold stress	107
Mannitol			
E. coli mt1D (Mannitol-1-phosphate dehydro genase) driven by CaMV 35S promoter	Tobacco	Better fresh weight, plant height and flowering under 250 mM NaCl for 30 days	108
E. coli mt1D	*Arabidopsis*	Transgenic seeds were able to germinate in up to 400 mM NaCl, whereas control seeds ceased to germinate at 100 mM NaCl.	109
E. coli mt1D	Tobacco	Better salt stress tolerance. Non-stressed transgenic plants were 20-25% smaller; Mannitol contributed only to 30-40% of the osmotic adjustment	110
E. coli mt1D	*Triticum aestivum* L.	Only 8% biomass reduction as compared to 56% reduction in control plants under 150 mM NaCl stress. High level of mannitol accumulation	111

		causes stunted growth and sterility	
Celery mannose 6-P reductase driven by CaMV 35S promoter	*Arabidopsis*	Enhanced salt tolerance in terms of growth, flowering and seed production in soil irrigated with 300 mM NaCl	112
D-Ononitol			
Ice plant Myo-inositol O-methyl transferase (*IMT1*)	Tobacco	Photosynthetic CO_2 fixation was slightly better under drought and salinity stress; faster recovery	113
Sorbitol			
Apple Stpd1 (sorbitol-6-phosphate dehydrogenase) driven by CaMV 35S promoter	Japanese persimmon, *Diospyros kaki* Thunb.	Tolerance in Fv/Fm ratio under NaCl stress	114

Genetically engineered overproduction of compatible osmolytes in transgenic plants such as *Arabidopsis*, tobacco, rice, wheat and *Brassica* has also been shown to enhance stress tolerance at the vegetative stage, as measured by germination, seedling growth, survival, recovery, and photosystem II yield (Table 1). Only in a few cases was salinity-stress tolerance of transgenics examined at the reproductive stage of the plant (94, 101, 108, 111, 112). In most cases, the contribution of the engineered osmoprotectant concentration to osmotic adjustment was not measured, or its contribution to osmotic adjustment was low. Abiotic stress tolerance of these transgenics was attributed to the osmoprotectant effect of these solutes (Table 1). Further, compartmentation of these osmoprotectants may also be required for enhanced tolerance. For example, transgenic rice plants that overexpress choline oxidase targeted to chloroplasts showed better tolerance to photoinhibition under salt and low-temperature stress than did plants overexpressing choline oxidase targeted to the cytosol (95). Often, engineered osmoprotectant overaccumulation results in impaired plant growth and development even under the nonstress environment. Transgenic tobacco plants overaccumulating mannitol (110), sorbitol (115) or trehalose (116), showed stunted growth. Often, engineered alterations in osmoprotectant accumulation results in infertility, depending on the concentration of osmoprotectant (111, 115). The use of a stress-inducible promoter to overexpress osmoprotectant biosynthesis helps in overcoming the growth defects while protecting the plants during osmotic stress (106).

Although transgenic tobacco overexpressing the myo-inositol O-methyl transferase gene accumulated D-ononitol in the cytosol up to 600 mM during salt stress, D-ononitol did not enter the vacuole (113). Hence, further understanding of the metabolic flux and compartmentation of osmoprotectants will help in precisely engineering osmoprotectant metabolism in plants for salt-stress tolerance.

REGULATION OF OSMOPROTECTANT METABOLISM

Evidence from genetic analysis, gene expression and transgenic studies show that osmoprotectant biosynthesis and accumulation in appropriate cellular organelles is critical for plant salt tolerance. However, the signaling cascades that regulate the osmoprotectant biosynthesis and catabolism during salt and other osmotic stress in higher plants are poorly understood. A signaling cascade similar to that of the yeast mitogen-activated protein kinase-high osmotic glycerol 1 (MAPK-HOG1) pathway may be involved in regulation of osmoprotectant biosynthesis (3, 19). *Arabidopsis AtHK1*, a putative osmosensory two-component hybrid histidine kinase is implicated in osmosensing during salt stress. *AtHK1* expression is induced by salt stress, and it complements the yeast double mutant *sln1*Δ *sho1*Δ, which lacks osmosensors. Similar to the SLN1 osmosensor of yeast, AtHK1 is probably active at low osmolarity and may inactivate a response regulator by phosphorylation. High osmolarity caused by salt stress may inactivate AtHK1, which results in the accumulation of the active form of the nonphosphorylated response regulator and may activate osmolyte biosynthesis in plants by activating the MAPK pathways (19). Moreover, constitutive overexpression of a dominant-negative mutated form of *AtHK1* in transgenic *Arabidopsis* resulted in enhanced tolerance to salt and drought stress (117). Results from complementation analysis in yeast and transgenic *Arabidopsis* suggest that AtHK1 may act as an osmosensor in *Arabidopsis*. Determination of the *in vivo* role of higher plant putative sensory kinases and the identification of signaling intermediates and targets will shed more light on salt-stress signaling.

Genetic analysis of ABA-deficient mutants *los6/aba1* and *los5/aba3* of *Arabidopsis* revealed that proline biosynthesis during osmotic stress is regulated by ABA, because salt and other abiotic stress induction of *P5CS* gene expression is either

diminished or blocked in these mutants (118, 119). In *Arabidopsis* and *Medicago truncatula*, of the 2 *P5CS* genes, the expression of only one gene is regulated by NaCl and osmotic stress (120, 121), which suggests that the promoters of these genes are differentially activated by developmental and osmotic stress cues. Biochemical analysis implicates phospholipase D (PLD) as a negative regulator of proline biosynthesis in *Arabidopsis* (122). Recent studies have shown that proline can act as signaling molecule to autoregulate the proline concentration and induce salt-stress-responsive proteins. In the desert plant *Pancratium maritimum* L., severe salt stress resulted in an inhibition of antioxidative enzymes such as catalase and peroxidase. Exogenous application of proline helped to maintain the activities of these enzymes and also upregulated several salt-stress-responsive dehydrin proteins (123). Microarray and RNA gel blot analyses have shown that 21 proline inducible genes have the proline- or hypo-osmolarity-responsive element (PRE, ACTCAT) in their promoter (124, 125). Transient activation analysis of a PRE-containing promoter led to the identification of 4 bZIP transcription factors that may regulate proline dehydrogenase and other proline- or hypo-osmolarity-responsive genes in *Arabidopsis* (126). Understanding the signaling events that regulate osmoprotectant metabolism during stress and recovery will be useful in improving salt and osmotic stress tolerance of crop plants.

LEA-TYPE PROTEINS

Late-embryogenesis-abundant (LEA) proteins are synthesized and stored in maturing seeds and are necessary for the desiccation tolerance of seeds. LEA-type proteins coding genes are called dehydrins, 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 (3, 127). LEA proteins are induced at higher levels by salt or ABA in salt-tolerant indica rice varieties than in salt-sensitive rice varieties (128). In higher plants, osmotic stress and ABA induce several LEA-type proteins in vegetative tissues. The expression levels of LEA proteins are correlated with desiccation tolerance in vegetative tissues, pollen and seeds (129, 130). LEA proteins are rich in hydrophilic amino acids and are vary stable. The proposed functions of LEA proteins under stress are to i) protect the cellular structure by acting as a hydration buffer,

ii) protect proteins and membranes, and iii) renature denatured proteins (129, 130). Genetically engineered rice plants constitutively overexpressing a barley *LEA* gene (*HVA1*) driven by rice actin-1 promoter showed better salt (200 mM NaCl) and drought stress tolerance and faster recovery once the stress was removed. Wilting, dying of old leaves and necrosis of young leaves were delayed in transgenic rice as compared with control plants under both salt and water stress (131).

Transcriptional Regulation of *LEA/COR* Genes

ABA regulates several aspects of plant development, including seed development, desiccation tolerance of seeds and seed dormancy and plays a crucial role in abiotic and biotic stress tolerance of plants. Genetic analysis of ABA-deficient mutants established the essentiality of ABA signaling in stomatal control of transpiration (31). As discussed earlier, since the rate of transpiration determines the amount of salt transport into shoots, stomatal regulation by ABA is an important trait of plant salt tolerance. Salt and osmotic stress regulation of *LEA* gene expression is mediated by both ABA-dependent and –independent signaling pathways. Both the pathways appear to employ Ca^{2+} signaling, at least in part, to induce *LEA* gene expression during salinity and osmotic stress (3, 127). Northern analysis of *COR* gene expression in ABA-deficient mutants, namely *los5/aba3* and *los6/aba1* of *Arabidopsis*, showed that ABA plays a pivotal role in salt and osmotic stress-regulated gene expression. The expression of *RD29A*, *RD22*, *COR15A* and *COR47* was severely reduced or completely blocked in the *los5* mutant (118), whereas in *los6*, the expression of *RD29A*, *RD19*, *COR15A*, *COR47* and *KIN1* was lower than that in wild-type plants (119).

Promoters of *LEA/COR* genes contain dehydration-responsive elements/C-repeat (DRE/CRT), ABA-responsive elements (ABREs), MYC recognition sequence (MYCRS) and/or MYB recognition sequence (MYBRS) *cis*-elements. The regulation of gene expression through DRE/CRT *cis*-elements appears to be mainly ABA independent, whereas ABRE and MYB/MYC element-controlled gene expression is ABA dependent (127, 132). However, recent studies have shown that cross-talk exists between the ABA-dependent and -independent pathways. For example, *RD29A* expression depends on both *DRE* and *ABRE* elements (133), and ABA can also induce the expression of C-repeat

binding proteins, CBF1-CBF3 (134). Salt-stress signaling through Ca^{2+} and ABA mediate the expression of *LEA* genes by transcription factors that activate *CRT*, *ABRE* and *MYC/MYB cis*-elements (Fig. 4).

Calcium Sensor Proteins

An earlier section of this chapter described the role of ABA in regulating cytosolic Ca^{2+} signatures during salinity. Genetic and biochemical evidence show that ABA-mediated *COR* gene expression is regulated by Ca^{2+} signaling. In addition to SOS3, salt stress-induced Ca^{2+} oscillations may also be perceived by Ca^{2+}-dependent protein kinases (CDPKs) and calmodulins (CaMs). *Arabidopsis AtCDPK1* and *AtCDPK2* are induced by salt and drought stress (135). In rice, salt, drought and cold stress induce the expression of *OsCDPK7* (136). In *Mesembryanthemum crystallinum* (common ice plant), salinity and dehydration regulate myristoylation and localization of a CDPK (McCPK1) into the plasma membrane. Upon dehydration, McCPK1 changes its cellular localization from the plasma membrane to the nucleus, endoplasmic reticulum and actin microfilaments (137). McCDPK1 phosphorylates the McCDPK1 substrate protein 1 (CSP1) *in vitro* in a Ca^{2+}-dependent manner, and salt stress induces co-localization of McCDPK1 and CSP1 in the nucleus of ice plants (138). CDPKs transduce salt stress and ABA-induced Ca^{2+} signals to regulate the expression of LEA-type genes (136, 139). Transient expression analysis in maize protoplasts showed that an increase in cytosolic Ca^{2+} concentration activates CDPKs that induce the stress-responsive *HVA1* promoter, which is under the negative control of ABI1 protein phosphatase 2C (139). Overexpression analysis also confirmed the regulatory role of CDPKs in salinity-induced LEA gene expression. Transgenic rice overexpressing *OsCDPK7* showed enhanced induction of a *LEA*-type gene (*RAB16A*) and salt/drought tolerance, whereas antisense transgenic plants were hypersensitive to salt/drought stress (136).

CaMs may act as negative regulators of salt stress-induced Ca^{2+} signatures. Overexpression of *CaM3* in *Arabidopsis* repressed the expression of *COR* genes (*RD29A* and *COR6.6*) (140). The expression of *COR* genes is mediated by Ca^{2+} signals (141). Ca-ATPases mediate Ca^{2+} efflux from the cytoplasm and thus regulate the magnitude and duration of cytosolic Ca^{2+} oscillations. Endoplasmic reticulum Ca-ATPase (ACA2) has

been shown to be activated by CaM and inhibited by CDPK (142). Salinity, dehydration and cold stress-inducible *AtCaMBP25* (*Arabidopsis thaliana* calmodulin (*CaM*)-binding protein of 25 kDa) binds to a canonical CaM in a Ca^{2+}-dependent manner. Transgenic plants overexpressing *AtCaMBP25* showed hypersensitivity to salt and osmotic stress, whereas antisense *AtCaMBP25* transgenic plants were more tolerant to these stresses than the wild type. These results suggest that AtCaMBP25 may function as a negative effector of salt and osmotic stress signaling (143). The differences in affinity of SOS3, SCaBPs, CDPKs and CaM for Ca^{2+} may determine the operation of specific signaling cascades and interactions. Thus, *LEA/COR* gene expression is regulated by the balance between the activities of CDPKs and CaMs. Ca-CaMs may also regulate cytoplasmic receptor-like kinases during salt and abiotic stress signaling. Salt-, cold- and H_2O_2-inducible CaM binding cytoplasmic receptor-like kinase 1 (CRCK1) has been cloned from alfalfa (144). Transcriptome analyses showed the induction of receptor-like kinase genes in *Arabidopsis* under salt stress (14, 15). However, the roles of these proteins in salt-stress sensing and their targets are unknown.

Basic Lucine-Zipper-Family Transcription Factors

ABA-dependent expression of *COR* genes under osmotic stress is regulated by basic leucine-zipper (bZIP) (145) and MYB/MYC-type transcription factors (Fig. 4) (146). Salt, drought and ABA upregulate the expression of *Arabidopsis* bZIP transcription factors such as *ABREB1* (ABA-responsive element binding protein 1 = *ABF2*) and *ABREB2* (= *ABF4*) genes. These transcription factors have been shown to induce *RD29B* promoter-*GUS* in leaf protoplasts of wild-type *Arabidopsis* but not in *aba2* (ABA-deficient) and *abi1* (ABA-insensitive) mutants. The induction of *RD29B-GUS* by *ABREB*s is enhanced in an *era1* (enhanced response to ABA) mutant. This evidence suggests that ABA is necessary for the expression and activation of ABREB1 and ABREB2, which in turn regulate *COR* gene expression (145). Constitutive overexpression of *ABF3* and *ABREB2 (=ABF4)* in *Arabidopsis* enhanced the expression level of target *LEA* genes (*RAB18* and *RD29B*). These transgenic plants showed hypersensitivity to ABA, sugar and salt stress during germination but enhanced drought tolerance at the seedling stage (147).

MYB/MYC-Type Transcription Factors

MYB/MYC-type transcription factors such as AtMYC2 (=RD22BP1) and AtMYB2 regulate *LEA* gene expression in *Arabidopsis* during osmotic stress (Fig. 4). Transgenic *Arabidopsis* plants overexpressing *AtMYC2* and *AtMYB2* showed constitutive expression of *RD22* and *AtADH*, and the expression levels were further increased with ABA treatment. The expression of *RD22* and *AtADH* genes is impaired in the *atmyc2* mutant. Transgenic *Arabidopsis* plants overexpressing *AtMYC2* and *AtMYB2* showed enhanced osmotic stress tolerance, as measured by electrolyte leakage from cells (146), although their salt stress tolerance is not known. Overexpression of ABA- and abiotic stress-inducible *Craterostigma plantagineum MYB10* enhanced salinity and desiccation tolerance of transgenic *Arabidopsis* plants. These transgenics also showed ABA hypersensitivity and altered sugar sensing. *In vitro* promoter binding assay showed that CpMYB10 binds to the *LEA Cp11-24* promoter (148).

C-repeat Binding Proteins

*CBF*s (C-repeat binding proteins) or dehydration-responsive-element binding proteins (*DREB*s) belong to the EREBP/AP2 domain transcription factor family. CBFs activate the expression of *LEA/COR* genes through *DRE*/*CRT cis*-elements in response to abiotic stress. *Arabidopsis DREB*s are classified into 2 classes: *DREB1* (*DREB1A=CBF3*, *DREB1B=CBF1*, *DREB1C=CBF2* and *CBF4*) and *DREB2* (*DREB2A* & *DREB2B*). The expression of *CBF1*, *CBF2* and *CBF3* is induced by cold stress, whereas that of *CBF4* is induced by drought stress. The expression of *DREB2A and DREB2B* is induced by dehydration and salt stress (132, 149, 150). Similar to *Arabidopsis DREB2*, rice *OsDREB2A* is induced by dehydration and salt stress (151). Osmotic stress-induced expression of *CBF4* appears to be mainly mediated by ABA (150). ABA has been shown to induce *CBF1*, *CBF2* and *CBF3*, although their ABA-induced expression level is significantly lower than with cold stress (134). Transgenic plants overexpressing *CBF* (*CBF1, 3 & 4*) genes showed constitutive activation of *DRE/CRT* cis-element-dependent *COR* gene expression (149-153). Transcriptional activation of *COR* genes by CBF transcription factors is conserved across plant species such as *Arabidopsis*, wheat, *B. napus* (154), barley and rice (151). Transcriptome analysis of *CBF*-overexpressing

transgenic *Arabidopsis* showed that approximately 13 *LEA/dehydrin* genes are under the transcriptional control of CBFs (155). Recently, ICE1 (inducer of CBF expression 1), a MYC-type basic helix-loop-helix transcription factor, as an upstream regulator of CBFs under cold stress was identified in *Arabidopsis* (156) (Fig. 4). Upstream transcription factors that regulate the expression of *DREB2/CBFs* during salt stress have yet to be identified.

In tobacco *Tsi1* (tobacco-stress-induced-gene 1; a member of the EREBP/AP2 transcription factor family), gene expression is rapidly induced by salt but not drought or ABA. The overexpression of *TSI1* in tobacco enhanced the retention of chlorophyll content when leaves were floated in 400 mM NaCl solution for 48 or 72 h (157). Further detailed studies are needed to identify the targets of TSI1.

Transgenic *Arabidopsis* overexpressing *CBF1 or CBF3* driven by the CaMV35S promoter or *CBF3* expression under the transcriptional control of the stress-responsive *RD29A* promoter showed enhanced tolerance to salt, drought and freezing stress (149, 152-154). Transgenic wheat plants expressing *RD29A::CBF3* showed enhanced osmotic stress tolerance (158). Overexpression of the rice *OsDREB1A* gene in *Arabidopsis* resulted in the activation of target *LEA* genes and conferred salt and other abiotic stress tolerance (151). Constitutive overexpression of *CBF1 or CBF3* resulted in growth abnormalities of the transgenic plants (149, 152-154, 159, 160). This problem has been overcome by the use of a stress-responsive promoter to drive the expression of *CBF*s (153, 158). Salt and abiotic stress tolerance of *CBF*-overexpressing transgenic plants was attributed to the enhanced expression of *LEA* genes (153, 154), accumulation of compatible osmolytes (161) and enhanced oxidative stress tolerance (159, 160). Genome-wide expression analysis showed that *CBF* overexpression also induces transcription factors such as AP2 domain proteins (*RAP2.1* and *RAP2.6*), putative zinc finger protein, and R2R3-MYB73 (155), which might regulate genes involved in osmolyte biosynthesis and antioxidant defense. This evidence shows that the expression of several genes can be manipulated in transgenic plants engineered with a single CBF transcription factor and enhanced expression of *LEA* genes is critical for salt and other abiotic stress tolerance.

OXIDATIVE STRESS MANAGEMENT

Reactive oxygen species (ROS), namely, superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) are produced in aerobic cellular processes such as mitochondrial and chloroplast electron transport and oxidation of glycolate (photorespiration), xanthine, and glucose. ROS cause oxidative damage to membrane lipids, proteins and nucleic acids. Hence, organisms have evolved various antioxidants and detoxifying enzymes to efficiently scavenge ROS. Antioxidant enzymes employed by plants are ascorbate, glutathione, α-tocopherol and carotenoids, whereas detoxifying enzymes include superoxide dismutase (SOD), catalase, peroxidase and enzymes of the ascarbate-glutothione cycle. SOD converts superoxide to H_2O_2, which is detoxified to water and oxygen by the catalase and/or ascorbate-glutathione cycle. Salt stress induces the accumulation of ROS and enhances the expression of ROS-detoxifying enzymes (5, 162, 163, 164). Alleviation of oxidative damage by scavenging ROS is an important strategy of plants to tolerate stress (3, 165, 166, 167). Hence, several efforts have been made to improve salt tolerance by engineering ROS-detoxifying enzymes.

Transgenic plants overexpressing ROS-scavenging enzymes such as SOD (168), ascorbate peroxidase (APX) (169) and glutathione S-transferase/glutathione peroxidase (GST/GPX) (170, 171) showed increased tolerance to osmotic, temperature and oxidative stress. The overexpression of the tobacco *NtGST/GPX* gene in transgenic tobacco plants improved salt- and chilling-stress tolerance because of enhanced ROS scavenging and prevention of membrane damage (170, 171). Transgenic tobacco plants overexpressing *AtAPX* targeted to the chloroplasts showed enhanced tolerance to salinity and oxidative stress (172). The *Arabidopsis pst1* (*photoautotrophic salt tolerance 1*) mutant is more tolerant to salt stress than is the wild type. The salt tolerance of this mutant was attributed to higher activities of SOD and APX than in the wild-type *Arabidopsis* (173). These evidences show that ROS detoxification is an important trait of plant salt tolerance.

Salt stress (5, 162) and ABA (174, 175) induce enhanced production of H_2O_2. ABA-dependent ROS production is catalyzed by NADPH oxidase, as revealed by analysis of the *atrbohD/F* double mutant of *Arabidopsis*, which is impaired in ABA-induced ROS production (176). ABA-elicited H_2O_2 production is negatively regulated by the ABI2 protein (177). H_2O_2 acts as a systemic molecule in regulating the expression of

GST and *GPX* genes (178). The accumulation of H_2O_2 in leaves of catalase-deficient tobacco plants was sufficient to induce the production of defense proteins (GPX, PR-1) locally as well as systemically (179). Promoter analysis of the salt stress-inducible *Citrus sinensis GPX1* (phospholipid hydroperoxide) gene suggests that *GPX1* upregulation under salinity is mediated by H_2O_2 but not superoxide (172). Promoters of genes that encode ROS-detoxifying enzymes contain antioxidant-responsive elements (ARE), ABA-responsive elements (ABRE), NF-κB redox-regulated transcription factor recognition sequences, heat shock elements (HSE) and redox-regulated transcription factor Y-box *cis*-elements (180). Hence, ABA, as well as H_2O_2, may act as a second messenger to regulate antioxidant defense genes during salinity. Oxidative stress signaling is probably mediated by the MAPK cascade in plants (180, 181).

Pyramiding of chloroplastic and mitochondrial Mn-SOD in alfalfa resulted in lower biomass production compared with that in transgenic plants expressing either of the Mn-SODs (182). Engineered alterations in antioxidant systems may alter the pool size of ROS, which are involved in developmental, biotic and abiotic stress signaling (175, 183). In field environments, crop plants often experience more than one biotic and abiotic stress. Critical evaluation of the engineered alterations in the antioxidant system on crop productivity in the normal environment as well as under multiple stress environments in field conditions and understanding the signaling components that regulate ROS detoxification during salinity is needed to use this trait for genetically engineering plant salt tolerance.

MAPK SIGNALING PATHWAY

ROS signaling in plants under various stresses is mediated by mitogen-activated protein kinase (MAPK) signaling pathways (184, 185). Salt stress triggers the activation and enhanced gene expression of MAPK signaling cascades, some components of which are common for both salt and ROS (181, 186). The *Arabidopsis* genome encodes approximately 60 MAPKKKs but only approximately 10 MAPKKs and 20 MAPKs (187). Hence, signals perceived by the 60 MAPKKKs must be transduced through 10 MAPKKs to 20 MAPKs. Thus, MAPK cascades offer potential nodes for stress, hormonal and developmental signal cross-talk. Salt stress activates *Arabidopsis*

AtMEKK1 (=MAPKKK) (188), AtMKK2 (=MAPKK) (189) and MAPKs (ATMPK3, ATMPK4 and ATMPK6) (190, 191). The active form of AtMEKK1 has been shown to activate AtMPK4 *in vitro* (192). Yeast 2-hybrid analysis, *in vitro* and *in vivo* protein kinase assays and analysis of *mkk2* null mutants have led to the identification of a MAPK signaling pathway consisting of AtMEKK1, AtMEK1/AtMKK2, and AtMPK4/AtMPK6 (188, 189, 191) that transduces salt and other abiotic stress signals in *Arabidopsis*. Transgenic *Arabidopsis* plants overexpressing *AtMKK2* showed constitutive AtMPK4 and AtMPK6 activity and enhanced salt (germination on 150 mM NaCl medium) and freezing tolerance, whereas *mkk2* mutant plants exhibited impaired activation of AtMPK4 and AtMPK6 and thus hypersensitivity to salt and cold stress (189). In addition to salinity, H_2O_2 activates AtMPK3 and AtMPK6 (193), probably through H_2O_2-activated ANP1 (=MAPKKK) (194). Transgenic tobacco plants overexpressing a constitutively active tobacco *ANP1* orthologue, *NPK1*, exhibited constitutive AtMPK3 and AtMPK6 activity and enhanced salt- (300 mM NaCl for 3 days), drought- and cold-stress tolerance (194).

Gene expression analysis of *AtMKK2*- and *ANP1*-overexpressing transgenic *Arabidopsis* plants led to the identification of target genes of this MAPK pathway. Overexpression of the active form of ANP1 showed activation of the *GST6* and *HSP18.2* promoters but not the *RD29A* promoter. A single amino acid mutation in the ATP-binding site of ANP1 abolished the ANP1 effect on these promoters (194). Microarray analysis of the transcriptome profile of *MKK2*-overexpressing plants identified approximately 152 target genes. Upregulated genes include CBF2, RAV1, RAV2, MYB and WRKY transcription factors, which may further regulate the expression of sub-regulons (189).

The *Arabidopsis* MAPK phosphatase 1 (*mkp1)* mutant exhibits salinity tolerance but hypersensitivity to genotoxic stress induced by UV-C. In a yeast 2-hybrid screen, MKP1 interacted with AtMPK3, 4 and 6. Microarray analysis of *mkp1* revealed that AtMKP1 negatively regulates a putative Na^+/H^+ antiporter AT4G23700 (195). Hence, MKP1 may negatively regulate salt stress signaling through AtMPK4. *Arabidopsis* nucleoside diphosphate kinase 2 (AtNDPK2) has been shown to interact with and activate AtMPK3 and AtMPK6 in yeast 2-hybrid and transgenic *Arabidopsis* plants overexpressing *AtNDPK2*. Further, these transgenic plants accumulated lower levels of

ROS and showed enhanced tolerance to salinity and other abiotic stress. A deletion mutation of *AtNDPK2* impaired AtMPK3 and AtMPK6 activities. This evidence suggests that AtNDPK2 is a positive regulator of stress signaling through MAPK pathways (193). In rice, the gene expression as well as kinase activity of *OsMAPK5* is regulated by ABA and biotic and abiotic stresses such as salt, drought, wounding, and cold. Transgenic rice overexpressing *OsMAPK5* showed increased tolerance to several abiotic stresses, including salt stress (196). These evidences show that diverse abiotic stress signals converge at MAPK cascades to regulate stress tolerance. Thus, in *Arabidopsis*, MAPK cascades consisting of AtMEKK1/ANP1, AtMEK1/AtMKK2, and AtMPK3/AtMPK4/ AtMPK6 may transduce salt-stress signaling. These MAPK cascades are further fine-tuned by a negative regulator, AtMKP1, and a positive regulator, AtNDPK1 (Fig. 5).

MOLECULAR BREEDING

Selection for yield under field stress conditions across environments is time and labor consuming. Hence, the identification of component physiological traits of salt tolerance, which are linked to stress tolerance in yield, will enhance the pace of breeding programs. These physiological traits often are controlled by multiple genes and show continuous variation in segregating populations. These types of traits are called quantitative traits, and the regions of chromosomes controlling these traits are called quantitative trait loci (QTLs). Identifying QTLs with use of molecular markers is the primary step for marker-assisted breeding and candidate gene cloning. The application of molecular markers to identify QTLs for physiological traits has helped to identify QTLs linked to salt-stress tolerance in different plant species (Table 2).

Table 2. QTLs for salt-stress tolerance in different plant species. Some examples showing the number of QTLs, contribution of individual QTLs, and combined effect of QTLs on phenotypic variation.

Plant Species	Mapping population	Component trait of salt tolerance	No. of QTLs & their contribution	Reference
Rice	RIL	Na^+, K^+ uptake and concentration	16	197
Rice	RIL	Dry mass; Na^+/ K^+ ratio	11; Individual QTLs contributed to 6-19% variation	198

Rice	RIL	Na^+, K^+ absorption	2	199
Rice	F2-F3	Na^+, K^+ uptake and concentration	2 major (one each for 48.5% and 40.1% variation in Na^+ and K^+ concentration, respectively) + minors	200
Lycopersicon spp.	Two different F2 populations	Fruit weight Fruit No.	4 (Cross1), 6 (Cross2) 10 (Cross1), 6 (Cross2); contribution of individual QTLs vary from 6-25%	201
Lycopersicon spp.	Inbred backcross (BC1 selfed)	Salt tolerance during germination	7, All QTLs accounted for only 45% variation; individual QTLs contributed to 6.5-15.6% variation	202
Lycopersicon spp.	Inbred backcross (BC1 selfed)	Salt tolerance during vegetative stage	5QTLs, Individual QTLs contributed to 5.7-17.7% variation, with the combined effects being about 46% of the phenotypic variation	203
Arabidopsis	RIL	Salt tolerance during germination & seedling growth	11 (6 for germination explaining 32% variation +5 for vegetative growth explaining 38% variation); individual QTLs contributed to 5-14% variation	204

In tomato, a major QTL (*fwTG48-TG180*) that accounted for 58% variation in fruit weight under control conditions contributed to only 14% variation with salt stress. However, the same QTL contributed to 17% and 8% of the genotypic variation under control conditions and salt stress, respectively, in another F2 population. The detection of approximately 50% or more of QTLs for salt tolerance depends on the salinity stress (201). Thus, QTLs are stress sensitive, and proper regulation of gene expression is critical for salinity tolerance. Further differential sensitivity of different phonological phases of plant development to salinity stress is evident from the results of QTL analysis. QTLs associated with tolerance at germination differ from those of vegetative growth (202-204). QTL analyses clearly establishes that i) salt tolerance is governed by multiple genes; ii) the contribution of individual significant QTLs can vary from 5% to 50%, depending upon the complexity of the trait; iii) the stress responsiveness of QTLs indicates the crucial role of gene regulation during stress; and iv) QTLs for tolerance at

different phenological phases specify the changes in salt tolerance mechanism during plant development.

If a QTL can be considered as a cluster of related genes that may be under the transcriptional control of one or more regulatory genes, one or more QTLs may also be under the transcriptional control of a single regulatory gene. The identification of gene(s) contributing to major QTLs and genetic transfer (breeding/genetic engineering) of a single regulatory gene that controls the expression of several target genes will significantly enhance the pace of development of salt-tolerant crops.

CONCLUSIONS AND PROSPECTS

During the past decade, the applications of molecular tools such as gene disruption and transgenic approaches have significantly enhanced our knowledge of salt-stress tolerance. Significant progress has been made toward understanding salt-stress signaling that controls ion homeostasis and salt tolerance. The SOS pathway regulates ion homeostasis during salt stress in *Arabidopsis*. Salt-stress sensor-induced cytosolic Ca^{2+} signals are perceived by SOS3, which in turn activates the SOS2 kinase. The activated SOS2 kinase regulates sodium efflux and sequesters 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, components and targets of these signaling pathways are not yet understood. CBFs, bZIP, MYB and MYC types of transcription factors induce LEA gene expression during osmotic stress. Molecular, genetic and cell biological approaches to identify signaling components and biochemical characterization of signaling complexes will be required to further understand salt-stress signaling pathways and their use in crop improvement.

The transgenic approach demonstrates the possibilities of gene transfer across organisms and engineering salt tolerance by manipulating a single gene or a few genes. Genetic engineering of ion transporters has been shown to significantly enhance salt tolerance (65, 77, 78). Transgenic manipulation of signaling molecules and transcription factors will be advantageous, because engineering a single gene can change the

expression of several target genes involved in stress response and provide multiple abiotic stress tolerance (68, 149, 152, 153, 189, 196). Often, constitutive overexpression of signaling components, osmoprotectants and stress-responsive genes results in reduced plant size and other growth abnormalities, even under normal growth conditions. Kasuga et al. (1999) demonstrated that the use of a stress-responsive promoter could overcome this problem. Hence, the selection of stress-responsive and tissue-specific promoters for engineering the stress-tolerance trait is critical. The overexpression of osmoprotectant and antioxidant systems has been shown to protect transgenic plants from salt stress. Some of the osmoprotectants, such as polyols and trehalose, overproduced in transgenics, are often associated with growth defects and sterility. Engineering for antioxidant systems may alter the pool size of H_2O_2, a signaling molecule involved in developmental and stress signaling. Hence, careful examination is needed in employing these traits to engineer salt-tolerant crops.

Most of the transgenics discussed here are model plants, and stress tolerance was assessed at the vegetative phase of growth in controlled conditions for very short durations. Often, transgenic plants are not evaluated under realistic stress conditions (2). In most cases, very high salt-stress levels are applied to clearly show the survival of transgenic plants and death of control plants, rather than their productivity under long-term realistic salinity levels. Hence, the effect of stress in relation to plant ontogeny should be assessed at realistic stress levels and under combinations that occur in nature, by using transgenic crop plants in the field. The identification of QTLs for salt tolerance in different crops will be needed for precise molecular breeding for salt-tolerant crops. The application of marker-assisted selection to QTLs of major effects should help in improving salt tolerance of crop plants. In the near future, pyramiding regulatory genes controlling the various aspects of salt tolerance (i.e., ionic and osmotic homeostasis, and damage control) in a single transgenic plant is expected to yield salt-tolerant crop plants with a very high level of tolerance to salt and osmotic stress.

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Figure 1. Effect of salinity stress on reproductive development in rice cv. M202.
a) Seedling stage is more tolerant to salinity than reproductive stage; b) Spikelet number is more sensitive to salinity than spikelet fertility (This graph was drawn by using the data from Zeng and Shannon, 2000; one dS m^{-1} is approximately equal to 10mM NaCl).

Figure 2. SOS signaling pathway regulates ion homeostasis during salt stress in *Arabidopsis*.
The *S*alt *O*verly *S*ensitive 3 (SOS3) perceive the salt stress induced Ca^{2+} signals and activates SOS2 kinase. Activated SOS2 kinase phosphorylates SOS1, a plasma membrane Na^+/H^+ antiporter. Phosphorylated SOS1 transports Na^+ out of cytosol. The *SOS1* transcript level and perhaps Na^+ transport through Na^+ transporter HKT1 are also regulated by SOS3-dependent SOS2 Kinase. The SOS2 kinase also activates tonoplast Na^+/H^+ antiporter (NHX1) that sequester Na^+ into the vacuole and vacuolar H^+/Ca^{2+} antiporter (VCX1). Activation of NHX1 and VCX1 by SOS2 are SOS3-independent and probably regulated through SOS3-like Ca^{2+} Binding Proteins (SCaBPs). ABI1 regulate the gene expression of NHX1 through ABFs (ABA responsive element Binding Factors). ABI2 interact with SOS2 and negatively regulate ion homeostasis either by inhibiting SOS2 kinase activity or the activities of SOS2 targets.

Figure 3. Osmoprotectants metabolism.
Genes encoding for many of these enzymes have been employed for genetic engineering osmoprotectant accumulation in plants (Table 1).

Figure 4. Transcriptional regulation of *LEA/COR* genes during salt stress.
LEA/COR genes are activated MYC/MYB and bZIP type transcription factors mainly through ABA-dependent signaling. Salinity induced ABA accumulation may mediate the expression of *CBF*s, which in turn induce *LEA/COR* genes expression through DRE/CRT *cis*-elements during salinity. The ICE1, a myc-like bHLH transcription factor, regulates the transcription of CBFs during cold stress, while the upstream signaling events that regulate CBFs expression under osmotic and ABA stresses are not known. Ca^{2+}

signaling is positively regulated by CDPKs and negatively regulated by ABI1/2 protein phosphatase 2C, SCaBP5-PKS3 complex and CaMs (* = indicates post-translation activation requirement)

Figure 5. MAPK Signaling pathways during salt stress in *Arabidopsis*.
An unknown sensor perceives and transduces the salt stress signals through MAPK pathways. Salt stress sensors activate MAPK cascades either in an ABA and reactive oxygen species (ROS) dependent or independent pathway. Activated MAPK (ANP1 & AtMEKK1 = MAPKKK; AtMEK1=MAPKK; AtMPK3, 4 & 6 = MAPK) cascades regulate salt stress responsive genes and salt tolerance. AtNDPK2 is a positive regulator of AtMPK3 & 6, while AtMKP1 is a negative regulator of AtMPK4 and 6.