

## Standard Review

# Recent advances in salt stress biology – a review

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**Soil salinity is one of the major abiotic stresses that adversely affects crop productivity and quality. Hence developing salt tolerant crops is essential for sustaining food production. Understanding of the molecular basis of salt stress signaling and tolerance mechanisms are essential for breeding and genetic engineering of salt tolerance in crop plants. The modern approaches being used to impart salt tolerance involves exploitation of natural genetic variations and/or the generation of transgenic plants. This review discusses the challenges and opportunities provided by recently developed molecular tools in the development of salt tolerant crops.**

**Key words:** Soil salinity, plasma membrane antiporter (*AtSOS1*), *AtHKT1*, vacuolar antiporter (*AtNHX1*), compatible solutes, reactive oxygen species (ROS), antioxidants.

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## INTRODUCTION

Agricultural productivity is severely affected by soil salinity. Environmental stress due to salinity is one of the most serious factors limiting the productivity of agricultural crops, most of which are sensitive to the presence of high concentrations of salts in the soil. About 20% of irrigated agricultural land is adversely affected by salinity (Flowers and Yeo, 1995).

The problem of soil salinity is further aggravated through the use of poor quality water for irrigation and inadequate drainage. Soil type and environmental factors, such as vapor pressure deficit, radiation and temperature may also alter salt tolerance. In clay soils, improper ma-

nagement of salinity and subsequent accumulation of sodium salts may lead to soil sodicity. In sodic soils sodium binds to negatively charged clay particles, causing swelling and dispersal, thus making the soil less fit for crop growth (Chinnusamy et al., 2005). The loss of farmable land to salinization is in direct conflict with the needs of the world population, projected to increase by 1.5 billion in the next 20 years (Blumwald and Grover, 2006).

Adverse effects of salinity on plant growth may be due to ion cytotoxicity (mainly due to  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^-$ ) and osmotic stress (reviewed by Zhu, 2002). Metabolic imbalances caused by ion toxicity, osmotic stress and nutritional deficiency under saline conditions may also lead to oxidative stress (Zhu, 2002). Hence, engineering crops that are resistant to salinity stress is critical for sustaining food prod-

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uction and achieving future food security. However, progress in breeding for salt tolerant crops has been hampered by the lack of complete understanding of the molecular basis of salt tolerance and lack of availability of genes that confer salt tolerance.

Two major approaches being used to improve stress tolerance are: (1) Exploitation of natural genetic variations and (2) generation of transgenic plants with novel genes or altered expression levels of the existing genes. Zhang et al. (2004) and Zhu (2001, 2002) recently reviewed signaling and transcriptional control in plants under salt stress. In this review, we discuss the approaches that have led to increased salinity tolerance, with particular emphasis on ion homeostasis, synthesis of compatible solutes and oxidative stress management.

### Ion homeostasis

Plants respond to salinity using two different types of responses. Salt-sensitive plants restrict the uptake of salt and adjust their osmotic pressure through the synthesis of compatible solutes (e.g. proline, glycinebetaine, soluble sugars; Greenway and Munns, 1980). Salt-tolerant plants sequester and accumulate salt into the cell vacuoles, controlling the salt concentrations in the cytosol and maintaining a high cytosolic  $K^+/Na^+$  ratio in their cells (Glenn et al., 1999).

A high NaCl concentration in tissues is toxic for growth of glycophytes (Glenn et al., 1999). The alteration of ion ratios in plants could result from the influx of  $Na^+$  through pathways that also function in the uptake of  $K^+$  (Blumwald et al., 2000). The maintenance of a high cytosolic  $K^+/Na^+$  ratio and precise regulation of ion transport is critical for salt tolerance (Glenn et al., 1999). This can be achieved by extrusion of  $Na^+$  ions from the cell or vacuolar compartmentation of  $Na^+$  ions. Three classes of low-affinity  $K^+$  channels have been identified (Sanders, 1995), these are  $K^+$  Inward rectifying channels (KIRC);  $K^+$  outward rectifying channels (KORCs) and Voltage-independent cation channels (VIC).

$K^+$  outward rectifying channels (KORCs) could play a role in mediating the influx of  $Na^+$  into plant cells. These channels, which open during the depolarization of the plasma membrane, could mediate the efflux of  $K^+$  and the influx of  $Na^+$  ions.  $Na^+$  competes with  $K^+$  uptake through  $Na^+$  -  $K^+$  co-transporters and may also block the  $K^+$  specific transporters of root cells under salinity (Zhu, 2003). This could result in toxic levels of sodium as well as insufficient  $K^+$  concentration for enzymatic reactions and osmotic adjustment. The influx of  $Na^+$  is controlled by AtHKT1, a low affinity  $Na^+$  transporter (Rus et al., 2001; Uozumi et al., 2000). The knockout mutant (*hkt1*) from Arabidopsis suppressed  $Na^+$  accumulation and sodium hypersensitivity (Rus et al., 2001), suggesting that AtHKT1 is a salt tolerance determinant, while the efflux is

controlled by Salt Overly Sensitive1 (SOS1), a plasma membrane  $Na^+/H^+$  antiporter (Shi et al., 2000). This antiporter is powered by the operation of  $H^+$  -ATPase (Blumwald et al., 2000). In addition to its role as an antiporter, the plasma membrane  $Na^+/K^+$  SOS1 may act as a  $Na^+$  sensor (Zhu, 2003). The overexpression of *SOS1* improved salt tolerance in Arabidopsis (Shi et al., 2003) (Table.1).

The compartmentation of  $Na^+$  ions in vacuoles provides an efficient and cost effective mechanism to prevent the toxic effects of  $Na^+$  in the cytosol. The transport of  $Na^+$  into the vacuoles is mediated by a  $Na^+/H^+$  antiporter (*AtNHX1*) that is driven by the electrochemical gradient of protons generated by vacuolar  $H^+$ -translocating enzymes, the  $H^+$ -ATPase and the  $H^+$ -PPiase (Blumwald, 1987). The overexpression of *AtNHX1*, resulted in the generation of transgenic arabidopsis (Apse et al., 1999), tomato (Zhang and Blumwald, 2001), *Brassica napus* (Canola) (Zhang et al., 2001), rice (Ohta et al., 2002), tobacco (Wu et al., 2004), maize (Yin et al., 2004), tall fescue plants (Luming et al., 2006) that were not only able to grow in significantly higher salt concentration (200 mM NaCl) but could also flower and set fruit. Also transgenic plants over expressing AVP1, coding for the vacuolar  $H^+$ -pyrophosphatase, showed enhanced salt tolerance (Gaxiola et al., 2001).

### Synthesis/overexpression of compatible solutes

The cellular response of salt-tolerant organisms to both long- and short-term salinity stresses includes the synthesis and accumulation of a class of osmoprotective compounds known as compatible solutes. These relatively small organic molecules are not toxic to metabolism and include proline, glycinebetaine, polyols, sugar alcohols, and soluble sugars. These osmolytes stabilize proteins and cellular structures and can increase the osmotic pressure of the cell (Yancey et al., 1982). This response is homeostatic for cell water status, which is perturbed in the face of soil solutions containing higher amounts of NaCl and the consequent loss of water from the cell. Glycinebetaine and trehalose act as stabilizers of quaternary structure of proteins and highly ordered states of membranes. Mannitol serves as a free radical scavenger. It also stabilizes sub cellular structures (membranes and proteins), and buffers cellular redox potential under stress. Hence these organic osmolytes are also 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). Although enhanced synthesis and accumulation of compatible solutes under osmotic stress is well known, little is known about the signaling cascades that regulate compatible solute biosynthesis in hig

**Table 1.** Salt tolerant transgenic plants expressing genes involved in synthesis/over expression of ion transporters and compatible solutes.

Gene	Gene product	Source	Cellular role(s)	Target plant	Reference
<b>Ion transporters</b>					
<i>AtNHX1</i>	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Arabidopsis thaliana</i>	Na <sup>+</sup> vacuolar sequestration	<i>Arabidopsis</i>	Apse et al., 1999.
<i>AVP1</i>	Vacuolar H <sup>+</sup> pyrophosphatase	<i>A. thaliana</i>	Vacuolar acidification	<i>Arabidopsis</i>	Gaxiola et al., 2001
<i>AgNHX1</i>	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Atriplex gmelini</i>	Na <sup>+</sup> vacuolar sequestration	Rice	Ohta et al., 2002
<i>AtSOS1</i>	Plasma membrane Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>A. thaliana</i>	Na <sup>+</sup> extrusion	<i>Arabidopsis</i>	Shi et al., 2003.
<i>GhNHX1</i>	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Gossypium hirsutum</i>	Na <sup>+</sup> vacuolar sequestration	Tobacco	Wu et al., 2004.
<i>AtNHX1</i>	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>A. thaliana</i>	Na <sup>+</sup> vacuolar sequestration	<i>Festuca arundinacea</i>	Luming et al., 2006.
<b>Compatible solutes</b>					
<i>COX</i>	Choline oxidase	<i>Arthrobacter globiformis</i>	Glycinebetaine	<i>Arabidopsis</i>	Hayashi et al., 1997
<i>ProDH</i>	Proline dehydrogenase	<i>Arabidopsis thaliana</i>	Proline	<i>Arabidopsis</i>	Nanjo et al., 1999.
<i>EctA</i>	L-2,4-diaminobutyric acid acetyltransferase	<i>Halomonas elongata</i>	Ectoynne	Tobacco	Nakayama et al., 2000.
<i>betA</i>	Choline Dehydrogenase	<i>E. coli</i>	Glycinebetaine	Tobacco	Holmstrom et al., 2000.
<i>Stpd1</i>	Sorbitol-6-phosphate dehydrogenase	apple	Sorbitol	Japanese persimmon	Gao et al., 2001.
<i>BADH</i>	Betaine dehydrogenase	<i>Atriplex hortensis</i>	Glycinebetaine	Tomato	Jia et al., 2002.
<i>TPSP</i>	Trehalose- 6-phosphate phosphatase	<i>E. coli</i>	Trehalose	Rice	Garg et al., 2002.
<i>PmSDH1</i>	Sorbitol dehydrogenase	<i>Plantago major</i>	Sorbitol	<i>Plantago major</i>	Pommerrenig et al., 2007.

plants. Salt tolerance of transgenic tobacco engineered to over accumulate mannitol was first demonstrated by Tarczynski et al. (1993). The other examples of compatible solute genetic engineering includes the transformation of genes for Ectoynne synthesis with enzymes from the halophilic bacterium *Halomonas elongata* (Nakayama et al., 2000; Ono et al., 1999) and trehalose synthesis in potato (Yeo et al., 2000), rice (Garg et al., 2002), and sorbitol synthesis in plantago (Pommerrenig et al., 2007) (Table 1). Initial strategies aimed at engineering higher concentrations of proline began with the overexpression of genes encoding the enzymes pyrroline-5-carboxylate (P5C) synthetase (P5CS) and P5C reductase (P5CR), which catalyze the two steps between the substrate (glutamic acid) and the product (proline). P5CS overexpression in transgenic tobacco dramatically elevated free proline (Kishor et al., 1995). However there is strong evidence that free proline inhibits P5CS (Roosens et al., 1999). Hong et al. (2000) achieved a two-fold increase in free proline in tobacco plants by using a P5CS modified

by site directed mutagenesis. The procedure alleviated the feedback inhibition of P5CS activity by proline and resulted in improved germination and growth of seedlings under salt stress. Also, Nanjo et al. (1999) used antisense cDNA transformation to decrease ProDH (Proline dehydrogenase) expression in order to increase free proline levels.

The enhancement of glycinebetaine syntheses in target plants has received much attention (Rontein et al., 2002). In spinach and sugar beet which naturally accumulate glycinebetaine, the synthesis of this compound occurs in the chloroplast. The first oxidation to betaine aldehyde is catalyzed by choline mono-oxygenase (CMO). Betaine aldehyde oxidation to glycinebetaine is catalyzed by betaine aldehyde dehydrogenase (BADH) (Rathinasabapathi, 2000). In *Arthrobacter globiformis*, the two oxidation steps are catalyzed by one enzyme, choline oxidase (COD), which is encoded by the *codA* locus (Sakamoto and Murata, 2000). Hayashi et al. (1997) used choline oxidase of *A. globiformis* to engineer glycinebetaine syn-

thesis in *Arabidopsis* and subsequently tolerance to salinity during germination and seedling establishment was improved markedly in the transgenic lines. Huang et al (2000) used COX from *A. panescens*, which is homologous to the *A. globiformis* COD, to transform *arabidopsis*, *B. napus* and tobacco. In this set of experiments COX protein was directed to the cytoplasm and not to the chloroplast. Improvements in tolerance to salinity, drought and freezing were observed in some transgenics from all three species, but the tolerance was variable. The results offered the possibility that the protection offered by glycinebetaine is not only osmotic but also function as scavengers of oxygen radicals. This point was also raised by Bohnert and Shen (1999) and further supported by the results of Alia et al. (1999). The level of glycinebetaine production in transgenics could be limited by choline. A dramatic increase in glycinebetaine levels (to 580 mmol/g dry weight in *arabidopsis*) was achieved when the growth medium was supplemented with choline (Huang et al., 2000).

### Antioxidant protection

Stress induces production of reactive oxygen species (ROS) including superoxide radicals, hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $OH^\cdot$ ) and these ROS cause oxidative damage to different cellular components including membrane lipids, protein and nucleic acids (Halliwell and Gutteridge, 1986). Reduction of oxidative damage could provide enhanced plant resistance to salt stress. Plants use antioxidants such reduced glutathione (GSH) and different enzymes such as superoxide dismutases (SOD), CAT, APX, glutathione-S-transferases (GST) and glutathione peroxidases (GPX) to scavenge ROS.

Transgenic tobacco plants overexpressing both GST and GPX showed improved seed germination and seedling growth under stress (Roxas et al., 1997). A major function of glutathione in protection against oxidative stress is the reduction of  $H_2O_2$  (Foyer and Halliwell, 1976). Ruiz and Blumwald (2002) investigated the enzymatic pathways leading to glutathione synthesis during the response to salt stress of wild-type and salt-tolerant *B. napus* L. (Canola) plants overexpressing a vacuolar  $Na^+/H^+$  antiporter (Zhang et al., 2001).

Wild-type plants showed a marked increase in the activity of enzymes associated with cysteine synthesis (the crucial step for assimilation of reduced sulfur into organic compounds such as glutathione) resulting in a significant increase in GSH content. On the other hand, these activities were unchanged in the transgenic salt-tolerant plants and their GSH content did not change with salt stress. These results clearly showed that salt stress induced an increase in the assimilation of sulfur and the biosynthesis of cysteine and GSH in order to mitigate salt-induced oxidative stress.

### Conclusions

The evaluation of salt tolerance in transgenic experiments has mostly been carried out using a limited number of seedlings or mature plants under laboratory and/or greenhouse conditions different from what the plants would naturally be exposed to (e.g. high-salinity soils, high diurnal temperatures, presence of other sodic salts etc). Thus, the evaluation of field performance under salt stress is difficult because of the variability of salt levels under field conditions (Daniells et al., 2001; Richards, 1983) and the potential for interactions with other environmental factors (including soil fertility, temperature, light intensity and water loss through transpiration).

Conventional breeding programs for generation of salt-tolerant genotypes have met with limited success. This lack of success is due in part to breeders preferring to evaluate their genetic materials under ideal conditions and also, the use of constitutive promoters like the CaMV 35S promoter, ubiquitin and actin promoters (Grover et al., 2003). However, stress-induced or tissue-specific promoters result in a better phenotype than overexpressing the same genes under a constitutive promoter (Kasuga et al., 1999; Zhu et al., 1998). There is a clear and urgent need to begin to introduce these tolerance genes into crop plants, in addition to establishing gene stacking or gene pyramiding.

Although progress in improving stress tolerance has been slow, there are a number of reasons for optimism. These include recent developments in the area of plant molecular biology, specifically 1) the development of molecular markers and gene tagging methodologies (2) the complete sequencing of model plant genomes (3) the production of T-DNA insertional lines of *arabidopsis* for gene tagging (4) the availability of forward genetics tools (Tilling) (Colbert et al., 2001) and (5) the availability of microarray analysis tools offers advantages and solutions to the complex intriguing questions of salt resistance.

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