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Review

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Salt Tolerance in Plants - Transgenic Approaches

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Abstract

Salinity is one of the major limiting factors for agricultural productivity. In plants, accumulation of osmolytes plays a pivotal role in abiotic stress tolerance. Likewise, exclusion or compartmentation of Na^+ ions into vacuoles provides an efficient mechanism to avert deleterious effects of Na^+ in the cytosol. Both vacuolar and plasma membrane sodium transporters and H^+-ATPases can provide the necessary ion homeostasis. A variety of crop plants were engineered with respect to the synthesis of osmoprotectants and ion-compartmentation, but there are other cellular pathways involved in the salinity responses that are still not completely explored. Genomics approaches are increasingly used to identify genes and pathway changes involved in salt-tolerance. The new knowledge may be used via guided genetic engineering of multiple genes to create crop plants with significantly increased productivity in saline soils. This review surveys how plants deal with high salt conditions and how salt tolerance can be improved by transgenic approaches.

Key words: Salt stress tolerance, osmoregulation, genomic approaches, transgenics

Introduction

Plant productivity is severely affected by salt stress. The presence of high Na^+ and Cl^- concentrations and an altered water status leads to metabolic toxicity, membrane disorganization, generation of reactive oxygen species (ROS), inhibition of photosynthesis and altered nutrient acquisition (Bohnert et al. 2001; Zhu 2002). Salt-tolerant plants evolved specialized complex mechanisms to counteract deleterious effects of salinity. The strategies are diverse between halophyte and glycophyte plants (Glenn et al. 1999). There are various mechanisms reported in the literature by which plants can protect themselves from these stresses, such as accumulation of osmoprotectants, exclusion of ions, compartmentation of ions, transporter and symporter systems, water channels, chaperones, superoxide radical scavenging machinery and signaling molecules. Traditional breeding approaches have yet to yield remarkable success because of the complexity of stress tolerance traits and incompatibility barriers to transfer genes from wild species to the cultivated ones. Partial progress has been made in the genetic engineering of plants by introduction of genes associated with osmoprotectants, scavenging ROS and other stress-induced proteins/genes, which seems to be a driving path to improve salt tolerance.

Nevertheless, engineering osmolyte pathways together with different sodium and potassium transporters could perhaps provide the necessary ion homeostasis during salt stress (Apse and Blumwald, 2002). Also, high-throughput functional genomic studies allow the discovery of novel salt-responsive genes (Bohnert et al. 2001). Based on such studies, a number of salt-responsive genes as well as key regulators could be identified, which serve as a valuable gene pool for the introduction of these genes into crop plants.

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Functional aspects of salt tolerance mechanisms: an account of transgenic salt tolerant plants

Genes that encode the synthesis of osmoprotectants, detoxification enzymes, stress proteins/chaperonins, aquaporins/water channels, ion transporters and kinases identified during salt-stress responses among various model species via molecular physiological approaches are reviewed by several authors (Bohnert et al. 2001; Zhu 2002). Genetic engineering for stress tolerance was limited in the pre-genomics era by the limited availability of genes and specific promoters (Zhu et al. 1997). Now, it is possible to study many genes simultaneously on a genome wide-scale with respect to their structure and function. In the current review, we provide a comprehensive outline of transgenics developed so far for salt tolerance by using various classes of genes (osmolyte biosynthesis, antioxidants, stress proteins that protect cell integrity, ion homeostasis and signaling pathways).

1. Osmolyte biosynthesis

Plants have evolved highly sophisticated mechanisms for balancing osmotic strength of cells under salt stress conditions. They can avoid dehydration by synthesizing different organic osmolytes that are congruous with cellular functions and can help them as osmotic balancing agents. Plants accumulate a very narrow range of compounds such as proline, glycine betaine, and sugars such as sucrose, trehalose, fructans and sugar alcohols like sorbitol, mannitol, ononitol, pinitol etc. during osmotic stress (Bray 1997). The accumulation of various osmoprotectants was a target for plant genetic engineering to develop genotypes tolerant to salinity and drought stress conditions for more than a decade. In most of the cases, introduction of a single foreign gene into a transgenic plant has led to moderate increase in tolerance with modest accumulation of osmoprotectants. In this section, we focus on genetic engineering work of osmoprotectant synthesis with special emphasis on proline, glycine betaine, trehalose and sugar alcohols.

(i) Role of proline biosynthesis and catabolism in salt tolerance

All plants accumulate proline under abiotic stress conditions but the quantity may range from 2 to 100-folds depending on the species and the extent of stress. Accumulation of free proline under hyperosmotic conditions induced by high salinity or drought was well-documented (Delauney and Verma 1993, Kavi Kishor et al. 2005). In proline biosynthesis, pyrroline 5-carboxylate synthetase (P5CS) is a key enzyme, which catalyzes glutamate into pyrroline 5-carboxylate. Overexpression of a gene encoding for *Vigna aconitifolia* P5CS in transgenic tobacco plants resulted in high accumulation of proline up to 10-18 fold over control plants (Kavi Kishor et al. 1995). Transgenic plants exhibited better growth and enhanced biomass production under salt stress conditions. Zhu et al. (1998) introduced the same gene into rice under the control of a stress-inducible promoter and demonstrated that transgenic rice showed an increase in biomass under salt and water stress conditions with an increase of up to 2.5-fold more proline than control plants. Similarly, Sawahel and Hassan (2002) introduced this gene into wheat using *Agrobacterium* mediated gene transfer via indirect pollen system. These transgenic wheat plants displayed overproduction of proline and increased tolerance to salt. Since pyrroline 5-carboxylate (P5C) is an intermediate product between proline biosynthesis and proline catabolism, it is important to experiment with co-expression of pyrroline 5-carboxylate reductase (P5CR) along with P5CS that drives the proline biosynthesis reactions. P5CR was the first gene to be cloned in proline biosynthetic pathway by functional complementation of a *pro*C mutation in *Escherichia coli* with an expression library of soybean root nodule cDNA and was found to be osmoregulated (Delauney and Verma 1990). De Ronde et al. (2000) transformed soybean plants with a P5CR gene construct in an antisense direction controlled by an inducible heat shock promoter (IHSP). Reduction of the P5CR gene expression in antisense lines of soybean plants resulted in declined proline as well as protein synthesis. Antisense lines of transgenic soybeans did not withstand the osmotic stress due to decline in proline synthesis and accumulation. Low proline synthesis and accumulation in the transgenics resulted in a lower seed production than in control plants indicating that the antisense P5CR gene also negatively influenced seed production in soybean. In plants, proline is synthesized not only from glutamate but also from arginine/ornithine (Bryan 1990). In plants, ornithine is transaminated to glutamic semi aldehyde (GSA) by ornithine δ-aminotransferase (δ-OAT), which subsequently gets converted to proline via P5C (Delauney et al. 1993). In young seedlings of *Arabidopsis*, proline content, P5CS mRNA, δ-OAT mRNA as well as activity increased under salt stress conditions. These results provided hints that in *Arabidopsis*, ornithine as well as glutamate pathways play an important role together in proline accumulation during osmotic stress conditions. Proline dehydrogenase (PDH) is the rate-limiting step involved in proline catabolism. To investigate the role of proline catabolism in plants, Mani et al. (2002) generated

transgenic *Arabidopsis* with altered levels of PDH by sense (PDH-S plants) and antisense (PDH-AS plants) lines. The PDH transgenic plants did not show significant levels of osmotolerance under stress conditions. Nevertheless, applying exogenous proline increased tolerance to osmotic stress and proline was converted to glutamate in PDH-sense plants. However, transcription factors that induce proline biosynthetic pathway genes and their characterization is important to unravel the complex molecular mechanisms of proline accumulation.

(ii) Glycine betaine production and salt stress tolerance

Among different quarternary ammonium compounds, the biosynthetic pathway of glycine betaine was well studied in relation to salt tolerance. It occurs in bacteria, cyanobacteria, algae, fungi and many higher plants under osmotic stress conditions. In bacteria, choline gets converted to betaine directly by choline oxidase, encoded by the cod gene but this gene was not found in higher plants. The *cod* gene cloned from the soil bacterium *Arthrobactor globiformis* was fused with the 35S promoter, and a transit peptide from Rubisco small subunit gene (rbcS) was inserted between 35S promoter and *codA* gene (in order to target it to the chloroplast) and transferred into tobacco using Agrobacterium. The young transgenic plants survived at 400 mM NaCl for more than 30 days. Similar results were obtained earlier in diverse dicot species such as *Brassica*, *Arabidopsis*, tobacco (Huang et al. 2000; Hayashi et al. 1997) as well as in the monocot rice (Mohanty et al. 2002). In *E. coli*, a two-step pathway produces glycine betaine where choline dehydrogenase (CDH) oxidizes choline to betaine aldehyde, which is further converted to glycine betaine by betaine aldehyde dehydrogenase (BADH). Genes of *E. coli* involved in glycine betaine biosynthesis (CDH and BADH) were transferred and expressed in tobacco. Transgenic tobacco lines accumulated higher amounts of glycine betaine and exhibited higher biomass production and increased salt tolerance (Holmstrom et al. 2000). Alternatively, plants possess choline monooxygenase (CMO), a ferridoxin dependent soluble Rieske-type protein, which oxidizes choline to betaine aldehyde. Further, betaine aldehyde is converted to glycine betaine by betaine aldehyde dehydrogenase (BADH), which is a soluble NAD^+ dependent enzyme. CMO and BADH are stress inducible enzymes localized in the chloroplast stroma (Russell et al. 1998). A detailed review on glycine betaine synthesis in plants and its implications for enhancement of stress tolerance was published recently (Sakamoto and Murata 2002). A cDNA clone of BADH was isolated from *Atriplex hortensis* (halophyte) and was transferred using *A. tumifaciens* into a salt-sensitive tomato cultivar Bailichun under the control of 35S promoter. Transgenic tomato plants exhibited significantly higher levels of BADH transcript as well as enzyme activity in comparison to wild-type and also exhibited salt tolerance up to 120 mM NaCl. Since choline appears to be a limiting factor for betaine synthesis, the best possible way to increase choline synthesis is to up-regulate phosphoethanolamine-N-methyltransferase activity.

(iii) Sugars and sugar alcohols and their role in salinity tolerance

Some plants as well as bacteria accumulate sugars such as sucrose, trehalose, fructans and sugar alcohols like sorbitol, mannitol, ononitol, pinitol etc. during osmotic stress (Bray 1997). Crowe et al. (1998) presented evidence that sucrose can preserve the integrity of lipid bilayer of the membrane during dehydration. An interesting report was published on the improved tolerance to salinity of tobacco expressing yeast apoplastic invertase (Fukushima et al. 2001). The authors concluded that the changes in sucrose metabolism in transgenic plants protected the photosynthetic apparatus of the plants under salt stress conditions. Since invertases can cleave sucrose into glucose and fructose, invertases are expected to play a major role in generating high hexose pools, which could be used to synthesize D-ononitol, sorbitol, mannitol etc. This perhaps highlights the importance of sucrose degradation and genetic manipulation of relevant enzymes. The other sucrose cleaving enzyme sucrose synthase generates fructose and UDP-glucose, which opens the pathway for trehalose biosynthesis. Two stress-responsive sucrose synthase genes were isolated from the resurrection plant *Creterostigma platageneum* and characterized (Kleines et al. 1999). However, overexpression of these stress-inducible sucrose synthase genes and their role under salt stress remain to be elucidated.

Accumulation of a variety of polyhydroxylated sugar alcohols (polyols) under drought and salt stress conditions is reported in a number of organisms. Mannitol is synthesized from fructose-6-phosphate, while other sugar alcohols such as sorbitol, ononitol, pinitol and trehalose are synthesized from glucose-6-phosphate. Mannitol may not be synthesized in higher plants. Therefore, a bacterial gene encoding mannitol-1-phosphate dehydrogenase (*mtlD*) was isolated, introduced into tobacco that conferred salt tolerance with increased plant height and fresh weight under salinity stress (Tarczynski et al. 1993). Recently, Prabhavathi et al. (2002) overexpressed *mtlD* gene in egg-plants and the transgenics conferred salt tolerance. In sorbitol biosynthesis, genes encoding sorbitol-6-phosphate dehydrogenase (S6PDH) and sorbitol-6-phosphate phosphatase were considered to be im-

portant. Recently, a cDNA encoding NADP-dependent S6-PDH was isolated from apple and transferred into Japanese persimmon (*Diospyros kaki* Thunb. cv Jiro) via *Agrobacterium* mediated transformation. In the transgencis, sorbitol accumulation varied from 14.5 to 61.5 μmol g^{-1} that lead to enhanced salt stress tolerance. The enzyme myo-inositol O-methyl transferase (*imt1*) catalyzes myo-inositol to ononitol. The gene *imt1*, that catalyzes the first step in the synthesis

Table 1. Genes encoding for enzymes/proteins associated with salt tolerance

Osmolyte/Compound	Gene	Species	Cellular response
Proline biosynthesis	*P5CR*	Tobacco	Salt stress
	At-P5R (P5CR)	*Arabidopsis*	Salt stress
	P5CS	Tobacco	Salt stress
		Rice	Salt and water stress
		Wheat	Salt stress
		Carrot	Salt stress
	Anti-PDH	*Arabidopsis*	Freezing, Salt stress
	OAT	*Arabidopsis*	Salt stress
		Rice	Not induced by salt stress
Proline transport	*OsProT*	*Arabidopsis*	Induced by salt stress
	AtProT2	*L. esculentum*	Not induced by salt stress
	LeProT1		
	codA	*Arabidopsis*	Chilling, Salt stress
	codA	*Brassica*	Salt stress
	Chlcod	*Rice*	Salt, Cold stress
	CDH	Tobacco	Salt stress
	cox	*Arabidopsis*	Freezing, Salt, Drought stresses
Glycine betaine	*bet A*	Rice	Drought, Salt stresses
	betA/bet B	Tobacco	Low temperature, Salt stress
	CMO	Tobacco	Drought, Salt stresses
	BADH-1	Tomato	Salt stress
Sugars			
Fructan	*SacB*	Tobacco	Drought stress
Mannitol	*mtl1D*	Tobacco	Salt stress
	mtl1D	*Arabidopsis*	Seeds germinated under high salt stress
	mtl1D	*Arabidopsis*	Oxidative stress
D-Ononitol	*imt1*	Tobacco	Drought, Salt stresses
Sorbitol	*S6PDH*	*Diospyros kaki*	Salt stress
Trehalose	*ots A, otsB*	Tobacco	Drought stress
	TPS1	Tobacco	Drought stress
Aldose/Aldehyde reductase	*MsALR*	Tobacco	Tolerance to water deficit
Invertase	*Apoplastic invertase*	Tobacco	Salt stress
Stress Responsive Proteins			
LEA proteins			
	HVA1	Rice	Salt stress and water deficit
LEA proteins			Soil water deficit
LEA proteins	*HVA1*	Wheat	Freezing tolerance
	Cor15a	*Arabidopsis*	

Transporter	Gene	Species	Cellular response
Na^+/K^+-symporter (Na^+ influx system)	*AtHKT1*	*A. thaliana*	Salt stress
Na^+-K^+-symporter (Na^+ influx system)	*OsHKT1*	*Oryza sativa*	Salt stress
Na^+/K^+-symporter	*OsHKT2*	*Oryza sativa*	Salt stress
Na^+-H^+-dependent K^+ transporter	*EcHKT1*	*Eucalyptus*	Salt stress
Na^+-H^+-dependent K^+ transporter	*EcHKT2*	*calmodulensis*	Salt stress
	HvHKT1	*Hordeum vulgare*	
Na^+/H^+ antiporter (Plasma membrane)	*At SOS1*	*A.thaliana*	Salt stress
Na^+/H^+ antiporter (Vacuolar)	*AtNHX1*	*A.thaliana*	Salt stress
	AtNHX1	Tomato	
Na^+ transporter	*SAL1*	*A.thaliana*	Salt stress
Plasma membrane H^+-ATPase		*A.thaliana*	Salt stress
Vacuolar H^+ pyrophosphatase	*Avp1*	*A.thaliana* (expressed in yeast)	Salt stress
Ca^+, calmodulin dependent protein phosphatase calcineurin	Induces Na^+ efflux in plants or ENA1 in yeast	*Arabidopsis,* Yeast	Salt stress
K^+ uptake	*TRK1, TRK2*	Yeast	Salt stress
K^+ transporter	*AtKUP*	*A.thaliana*	Salt stress
	HAL1	Yeast, Rice	
	HAL1	Tomato	Salt stress
	HAL1	*A. thaliana*	Salt stress
K^+ transporter	*ATK1*	*Saccharomyces*	Salt stress
K^+ channel	*KAT1*	*A. thaliana* (guard cells)	Salt stress
$Ca2^+$ exchanger	*CAX1*	*A.thaliana*	Adaptation to chilling stress
$Ca2^+$ sensor	*Ca^{2+} sensor*	*A. thaliana*	Salt stress

of ononitol was introduced into tobacco and the transgenics exhibited higher accumulation of ononitol with enhanced salt and drought tolerance in comparison to their wild-types (Sheveleva et al. 1997). The gene encoding D-*myo*-inositol-3-phosphate synthase (*ins3ps*) in inositol biosynthesis was cloned from *Spirodela polyrhiza* and introduced into *Arabidopsis* (Smart and Flores 1997). Although *Arabidopsis* plants containing this gene accumulated higher levels of inositol, they did not confer salt tolerance. D-*myo*-inositol is not metabolically inert like other sugar alcohols but an important metabolite in the signal transduction pathways. Hence, inositol may not serve as a good compatible solute. Trehalose is accumulated in *E.coli*, yeast as well as in higher plants under osmotic stress conditions (Suprasanna 2003). Trehalose biosynthesis is controlled by the *otsA/B* locus in E.coli, which encodes trehalose 6-phosphate synthase (*otsA*). This enzyme catalyzes the formation of trehalose 6-phosphate from UDP-glucose and glucose 6-phosphate (Suprasanna 2003). Trehalose 6-phosphate phosphatase (*otsB*) then catalyzes the formation of trehalose from trehalose 6-phosphate. Tobacco plants transformed with trehalose 6-phosphate synthase subunit (*TPS1*) of yeast displayed improved performance under drought conditions (Pilon-Smits et al. 1998). Further, introduction of *TPS1* gene from *Saccharomyces cerevisiae* into potato resulted in drought tolerance (Yeo et al. 2000). It appears that transgenic rice plants producing trehalose also confer salt tolerance (Garg et al. 2002). It is thought that trehalose might replace the shell of water around macromolecules, preventing damaging effects during drying (Crowe et al. 1998). A plethora of genes associated with osmolyte biosynthesis and the corresponding transgenics that tolerated salt stress is shown in the table 1.

2. Antioxidant protection and detoxification pathways

At the physiological level, the effects of salt stress are multitude. Ion toxicity and water deficiency impair photosynthesis and produce reactive oxygen species (ROS). In addition to salt stress, several other stresses such as osmotic, high or low temperature, high light, heavy metals

trigger the production of excess of ROS. This in turn leads to phytotoxic reactions like lipid peroxidation, protein degradation and DNA mutation (Leprince et al. 2000). The degree of oxidative cellular damage in plants exposed to salt stress is controlled by the antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase and glutathione-S-transferase (GST). A correlation between the antioxidant capacity and NaCl tolerance was demonstrated in a number of crops such as pea (Hernández et al. 2000) and foxtail millet (Sreenivasulu et al. 1999; 2000). Noctor and Foyer (1998) provided a detailed account of the important antioxidants with reference to their biosynthesis, compartmentation and transport. Over expression of Mn-SOD (Bowler et al. 1991), Fe-SOD (McKersie et al. 1996) and chloroplastic Cu/Zn-SOD (Badawi et al. 2004) in transgenics lead to higher resistance against various stress conditions including salt. However, the results are often not correlated positively. In transgenic tobacco and tomato plants, overexpression of Cu/Zn superoxide dismutase failed to show protection against superoxide detoxification (Tepperman and Dunsmuir, 1990). Transgenic plants over expressing ascorbate peroxidase (Wang et al. 1999), glutathione peroxidase and glutathione reductase displayed better resistance to oxidative as well as salt stress conditions (Roxas et al. 1997). Over expression of glutathione-S-transferase and glutathione peroxidase in transgenic tobacco resulted in higher levels of glutathione and ascorbate than in wild type seedlings, and also exhibited reduced oxidative damage and higher degree of salt tolerance (Roxas et al. 2000). Tsugane et al. (1999) isolated a recessive *Arabidopsis* mutant designated as *pst1* (for photoautotrophic salt tolerance1) that grew photoautotrophically under salt stress. This mutant line showed enhanced active oxygen detoxification and 10-times more tolerance to methyl viologen than wild-type seedlings. There are few investigators who recorded up-regulation of phospholipid hydroperoxide glutathione peroxidase (PHGPX) transcripts in salt-stressed *Citrus* (Gueta-Dahan et al. 1997), salt-treated barley (Churin et al. 1999) and pea (Hernández et al. 2000). So far, there are no reports regarding the overexpression of PHGPX gene in plants. However, overexpression of PHGPX in rabbits inhibited hydroperoxide-induced oxidation. Besides SOD, APX, GPX and GST, catalases are also involved in detoxification/repair processes. Expression of wheat catalase cDNA in transgenic rice increased catalase activity by 4 to 15-fold and enhanced the tolerance against low temperature (Matsumura et al. 2002). C repeat/dehydration-responsive element binding factor 1 (CBF1) from *A. thaliana* cDNA driven by the 35S promoter, was transferred into tomato. These transgenic tomato plants displayed more resistance to osmotic stress than their corresponding wild types. Subtractive hybridization was used to isolate the responsive genes to heterologous CBF1 in transgenic tomato plants and catalase1 (*CATALASE1*) was characterized. While catalase activity increased, hydrogen peroxide concentration decreased in transgenic tomato plants when compared to wild type plants with or without water deficit stress. These results indicated that the heterologous *A. thaliana* CBF1 confers water deficit resistance in transgenic tomato plants. The aldose-aldehyde reductase family of genes might also function in a detoxification/repair pathway and prevent stress-induced damage. In support of this view, recently, Oberschall et al. (2000) showed the aldo-keto reductase functions in preventing lipid peroxidation in transgenics under drought conditions.

3. Protection of cell integrity

Late embryogenesis abundant (LEA) proteins are osmotically regulated proteins resulting from salt treatments, drought or cold temperatures and expressed abundantly during desiccation of seeds as well as in vegetative parts. LEA proteins are classified into 6 groups based on their sequence and kinetics (Dure 1993). Xu et al. (1996) transferred a cDNA clone encoding *Hordeum vulgare* LEA3 protein into rice plants with a constitutive promoter. This resulted in higher accumulation of this protein and also conferred salinity and drought tolerance. Although the mechanism involved in the action of this gene is not clear but the authors proposed that the improved salt tolerance might be due to stabilization of the cell structure. Recently, the barley LEA3 gene was expressed under the control of a stress-inducible promoter in a recalcitrant scented rice variety, Pusa Basmati-1, to increase the tolerance against salt stress. Third generation transgenic plants accumulated higher levels of LEA3 and showed increased salt stress tolerance by maintaining cell integrity and growth after the imposed salt as well as water-stress treatments, compared to the control plants (Rohila et al. 2002). Further, overexpression of the same gene in wheat plants reproduced the results, where transgenic wheat grew efficiently under osmotic stress conditions (Sivamani et al. 2000). Cheng et al. (2002) generated transgenic rice plants expressing a wheat LEA2 gene, and separately the wheat LEA1 gene. The second-generation transgenic plants expressed LEA2 (39 kDa) and LEA1 protein (25 kDa) in most of the lines and conferred increased tolerance to salt as well as drought stress conditions (Cheng et al. 2002). In general, group 2 LEA genes are referred to as dehydrins. Although LEA protein functions

are not described in detail, the proposed functions may include water retention, ion sequestration as well as chaperone activity. Recently, *HVA1*, a LEA gene from barley conferred dehydration tolerance in transgenic rice via cell membrane protection (Babu et al. 2004). But the molecular mechanisms associated with membrane protection are yet to be unravelled.

Heat shock proteins (HSPs) are a big family of genes that show induced expression under heat shock as well under osmotic stress conditions. HSPs act as molecular chaperones and probably function in protein folding and also protect proteins against denaturations. They are classified into high (HSP70, HSP80, HSP90, and HSP101) and low molecular weight HSPs (HSP17, HSP18 etc.). Although many transgenics raised for HSPs conferred thermotolerance (Hong and Vierling, 2000), there are few reports that pointed for their possible involvement in salt tolerance. HSP70 is induced by high salt stress in *Atriplex numularia* cells and its expression was not detected in unadapted cells (Zhu et al. 1993). To test the role of HSP70 (isolated from halotolerant cyanobacterium) during salt tolerance, transgenic *Nicotiana tabacum* were generated. They showed moderate photosynthetic activity and improved salt tolerance (Sugino et al. 1999). Sun et al. (2001) over expressed low molecular weight HSP17 in *A. thaliana* plants, that exhibited increased salt and drought tolerance. Further, these authors demonstrated chaperone activity for overproduced HSP17 protein *in vitro*. Osmotin and thaumatin are regulated under osmotic stress and were shown to confer tolerance to salt and drought stresses. Barthakur et al. (2001) over-expressed the osmotin gene under the control of constitutive CaMV 35S promoter in transgenic tobacco. They showed that overexpression of osmotin induced proline accumulation and retarded leaf senescence and improved germination under 200 mM NaCl in transgenics. Thaumatin protein strongly resembles that of osmotin. Overexpression of a gene that encodes a thaumatin like protein (PR-5) in rice conferred osmotic stress tolerance in transgenics (Datta et al. 1999).

4. Ion homeostasis and salt tolerance

(i) Sodium toxicity and transport

Nutrient levels and their availability in soils may vary in both time and space. Extreme nutrient conditions will cause deficiency as well as toxicity. Plants can tolerate salinity stress by salt exclusion at the plasma membrane or by salt inclusion at the vacuole (Rehman et al. 1998). Some of the halophytes (e.g. *Porteresia coarctata*) possess salt glands in their leaves and therefore, can exclude salts through them very easily. Adaptation of plants to Na^+ toxicity per se is not correlated with salt tolerance always but they need to cope with impaired nutrition especially K^+ acquisition (Greenway and Munns 1980). Salt-tolerant and sensitive lines may differ in ion uptake as well as pattern of accumulation of ions in different parts of a plant. While in salt-tolerant alfalfa line, more Cl^- accumulated in the plumules and radicals, in cotton more accumulation of Na^+ was noticed in the leaves. In barley, varietal differences in sodium and chloride uptake were reported (Rawson et al. 1988). Cytosloic Na^+ homeostasis must be maintained by removing ions either into the vacuole or to the outside. In *Saccharomyces pombe*, Jia et al. (1992) identified a new locus, sodium2 (*sod2*) in encoding an electroneutral Na^+/H^+ antiporter. Over expression of *sod2* increased Na^+ export capacity from the cells and conferred sodium tolerance. When *sod2* was disrupted, cells were not capable of exporting sodium. In plants, Na^+/H^+ antiporter is a candidate for sodium efflux (Apse et al. 1999). The tonoplast Na^+/H^+ exchanger, involved in sequestering Na^+ into plant vacuoles is expressed in roots and different parts of leaves. The highest activity of this protein is found in epidermal bladder cells of *M. crystallinum* (Barkla et al. 2002). *A. thaliana* salt overly sensitive (sos) mutant 1 (*Atsos1*) was shown to encode a plasma membrane Na^+/H^+ antiporter that has sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria and fungi (Shi et al. 2000). Further, a vacuolar Na^+/H^+ antiporter isolated from *Arabidopsis* and overexpressed in the same species resulted in tolerance to salt stress by promoting sustained growth and development under 200 mM NaCl treatment (Apse et al. 1999). Overexpression of this gene in *A. thaliana* displayed enhanced salt tolerance indicating the sequestration of Na^+ ions into vacuoles and protection of the cytosol. Further, a vacuolar Na^+/H^+ antiport gene from *A. thaliana* was transferred into *Brassica napus* (Zhang et al. 2001) and *L. esculentum* (Zhang and Blumwald 2001). Transgenic *Brassica* and tomato plants overexpressing *AtNHX1* were able to grow, flower, and produce seeds in the presence of 200 mM sodium chloride. In algae, though, Na^+ efflux is catalyzed by Na^+-ATPase (Balnokin and Popova 1994), such an evidence is lacking in higher plants. The vital house keeping functions for cellular metabolism, growth and ion homeostasis is carried out by vacuolar ATPase (V-ATPase) as well as by vacuolar H^+-Ppiase by creating proton motive force across the tonoplast of plant cells (Barkla and Pantoja 1996). Overexpression of the *A. thaliana* vacuolar H^+-pyrophosphatase (*AVP1*) conferred salt tolerance to the salt-sensitive *ena1* mutant of *Saccharomyces cerevisiae* (Gaxiola et al. 1999). Similarly, a vacuolar H^+-pyrophosphatase was over expressed in *Arabidopsis* that resulted in increasing the vacuolar proton gradient, which in turn lead to sequestration

of cations in the vacuole and increased drought and salt stress tolerance (Gaxiola et al. 2001). During salinity stress, the plant V-type H^+-ATPase mediates basic housekeeping functions as well as stress-induced NaCl sequestration. Exposure of plants to salt stress affected the expression of V-ATPase genes in a species in tissue specific manner (Binzel 1995) indicating requirement for enhanced activity of this enzyme for the compartmentation of sodium in response to osmotic stress (Barkla and Pantoja 1996). Evidence for coordinated expression and induction of genes that encode V-type H^+-ATPase subunit A and C isoforms in response to environmental cues like salinity stress was recently presented by Lehr et al. (1999) in sugar beet. Except the outward-rectifying K^+ channels (Czenpinski et al. 1997), cation efflux system has not yet been well characterized in plants. Hassidim et al. (1990) and Cooper et al. (1991) detected K^+/H^+ antiporter activity in *Atriplex* (halophyte) and oil-seed rape respectively in plasma membrane vesicles.

(ii) Potassium acquisition and transport

The perturbations in the ion ratios result from the influx of sodium through the pathways that function in the acquisition of potassium. Since plant proteins cannot discriminate between sodium and potassium, the key biochemical processes in the plant cell are inhibited by the competition of sodium for potassium-binding sites. Potassium is a major monovalent cationic component of plant cells and an essential nutrient. Potassium plays an important role not only in plant growth and development, but also in stomatal movements, enzyme activation and osmoregulation. The maintenance of a high cytosolic K^+/Na^+ concentration ratio is important for plant growth during salt stress conditions (Glenn et al. 1999). Rus et al. (2001) found that high affinity potassium transporter (AtHKT1) from *A. thaliana* functions as a selective Na^+ transporter and also mediates K^+ transport. Further, AtHKT1 identified as a regulator of Na^+ influx based on the capacity of *hkt1* mutants to suppress Na^+ accumulation and sodium hypersensitivity in a *sos3* mutant background. Laurie et al. (2002) introduced *HKT* into wheat in sense and antinsense direction and the transgenic lines expressing *HKT* transgene were tested for salinity responses under 200 mM NaCl. Transgenic lines showed enhanced growth under salinity and Na^+: K^+ ratios were reduced in salt-stressed transgenic tissue when compared with the control. K^+ uptake-deficient mutants of yeast were used to clone many K^+ channel homologues by complementation technique from *Arabidopsis* (Ko and Gaber 1991).

The yeast halotolerance gene (*HAL1*) facilitates K^+/Na^+ selectivity and salt tolerance of cells. Gaxiola et al. (1992) isolated a novel yeast gene, *HAL1*, which upon overexpression improved growth of yeast under salt stress, and disruption of this gene decreased salt tolerance capacity. Overexpression of *HAL1* gene in *A. thaliana* resulted in less sodium accumulation and promoted salt tolerance (Yang et al. 2001). Similarly, ectopic expression of *HAL1* in tomato plants enhanced growth of transgenics under salt stress by increasing K^+ content in both calli and leaves during the presence of salt (Rus et al. 2001). Sodium toxicity was counteracted by an increased potassium accumulation in those cells that overexpressed *HAL1* gene. Further, it also provided hints that *HAL1* probably could function in K^+/Na^+ selectivity under salt stress. Similarly, *HAL2* was cloned from yeast that encodes the 3',5'-bisphosphate nucleotidase, a salt-sensitive enzyme. Overexpression of this enzyme counteracted the decrease in activity produced by toxic levels of Na^+ or lithium (Glaser et al. 1993). Later, it was observed that this enzyme is involved in sulfate activation (Murguia et al. 1995). A homologue of *HAL2* was isolated from *Arabidopsis* (*SAL1*), that encoded not only 3',(2'),5'-bisphosphate nucleotidase but also inositol polyphosphate 1-phosphatase. Also, a rice *HAL2* like gene encoding a Ca^{2+} sensitive 3'(2'), 5'-diphosphonucleoside 3'(2') phosphohydrolase that complemented with yeast *met 22* and *E. coli cysQ* mutations was cloned by Peng and Verma (1995). The status of reduced sulfur in plant cells determines their ability to withstand stress. The above enzyme mediates the sulfur metabolic pathway without accumulation of any toxic intermediates (Goldschmidt et al. 1975). Over expression of both glutamine synthetase and *HAL2* genes in tobacco increased glutathione-an indicator of oxidative stress resistance and withstood the oxidative stress (Singh and Verma 2001). In yeast, *SAL1* restored the ability of a *hal2/met22* mutant to grow on sulfate as a sole sulfur source and increased salt tolerance (Quintero et al. 1996).

(iii) Signal transduction pathways of ionic transporters

Complexity of the stress responsive gene networks and upstream signal transduction pathways were explored recently by reverse genetic studies. Zhu et al. (1998) identified sos mutants, where mutant growth is impaired on media that are deficient in K^+ and particularly hypersensitive to Na^+ and Li^+ ions. Based on recent biochemical and physiological data the role of *sos1* in K^+ acquisition is indirect and SOS1 gene is identified as Na^+/H^+ antiporter, which maintains low concentration of Na^+ in cytoplasm by pumping Na^+ ions into acidic vacuoles (Shi et al. 2000). Subsequently, another locus, *SOS2*, and a third salt tolerance locus, *SOS3*, were identified in *Arabidopsis* by Zhu et al. (1998). *SOS2* locus has also been found to be necessary for K^+ nutrition since *sos2* mutants could not be grown in a medium with low

potassium and recently been identified as a serine/threonine type protein kinase with an N-terminal catalytic domain similar to that of the yeast SNF1 kinase (Liu et al. 2000). High concentration of Na^+ elicits a cytoplasmic calcium signal, which will be perceived by *SOS3*, a calcium binding protein. *SOS3* interacts with SOS2, a protein kinase, the regulatory domain is located in SOS2 gene. The calcium-dependent kinase pathway of *SOS3-SOS2* activates the Na^+/H^+ antiporter gene under excess of Na^+. It, therefore, appears that *SOS1*, *SOS2*, *SOS3* encode regulatory components controlling K^+ nutrition essential for salt tolerance in plants. Recently, Shi et al. (2002) isolated *sos4* mutant from *Arabidopsis* (hypersensitive to Na^+, K^+, and Li^+ ions) and demonstrated that *SOS4* encodes a pyridoxal kinase that is involved in the biosynthesis of pyridoxal-5-phosphate, an active form of vitamin B6.

Sodium tolerance in yeast is enhanced by activation of calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase that is required for modulation of the Na^+ efflux mechanism. Stress signalling through calcium- and calmodulin-dependent protein phosphatase calcineurin also plays a crucial role in ion homeostasis during salt stress (Pardo et al. 1998). A calcium sensor homolog appears to be required for salt tolerance in plants (Liu and Zhu 1998). Pardo et al. (1998) generated transgenic tobacco plants that are tolerant to high salinity stress by co-expressing yeast calcineurin. Recently, Park et al. (2001) isolated a *pmr* mutant and mapped the gene for P-type Ca^{2+}- ATPase, which maintains higher calcium in the mutant and confers salt tolerance through continuous activation of calcineurin. Liu and Zhu (1998) and Pardo et al. (1998) are of the opinion that this gene coordinates gene expression and activity of ion transporters to facilitate ion homeostasis during salt stress. In order to find the role of Ca^{2+}-dependent protein kinase (CDPK) in osmotic stress conditions, Saijo et al. (2000) overexpressed CDPK in rice and showed that it is a positive regulator commonly involved in the tolerance of salt and drought stress. To elucidate the function of shaggy-related protein kinase (GSK) in NaCl stress responses, Piao et al. (2001) generated transgenic *Arabidopsis* plants that overexpressed *AtGSK1* mRNA. Transgenic plants showed enhanced resistance to NaCl stress in whole plants with proper root growth. These plants accumulated higher Ca^{2+} levels (15 to 30%) in comparison to wild type plants when subjected to NaCl stress.

Genomic approaches of salt tolerance in crop plant improvement

Tolerance or sensitivity toward a particular stressful condition depends on the genetic and biochemical makeup of the species. Understanding the physiological and genetic mechanisms associated with salt stress tolerance by high-throughput genomic approaches is currently the promising approach. Using microarray technology, genes upregulated by abiotic stress were identified and are shown in the table 2 (Ozturk et al. 2002; Kawasaki et al. 2001). The large-scale partial sequencing of randomly selected clones (Expressed Sequence Tags; EST) from the cDNA libraries generated from salt-tolerant species provides an opportunity to fish out and catalog stress associated genes. The EST-based gene discovery program was extended to salt tolerant models such as *M. crystallinum*. So far, in this model plant 12,484 ESTs were produced from 14 different salt-treated cDNA libraries generated from various stages of tissue development. An identical approach has been advanced to generate ESTs that are related to salinity stress from glycophytes. Among dicots, approximately 3088 ESTs were

Table 2. Genes upregulated by salt stress - an index from microarray analysis

Functional class	Genes
Barley seedlings (3 week-old) exposed to 150 mM NaCl for 24h (Ozturk et al. 2002)	
Antioxidants	Glutathione-S-transferase (auxin-induced)
Jasmonate biosynthesis	Allene oxide synthase
Osmoprotectants	Proline rich protein, Δ^1-pyrroline-5-carboxylate synthetase
Photosynthetic	Photosystem II 10 K protein
Protein destination	Metallothionein-like protein type 2, aspartic proteinase transcription factor POU3A, acidic ribosomal protein
Regulatory	60S replicase associated polyprotein
Stress responsive genes	Heat shock protein DnaJ, lipid transfer protein cw 18,
	Late embryogenesis abundant like protein
Unknown	6 unknown genes

Rice seedlings exposed to 150mM NaCl for 15 min, 1h, 3h and 6h (Kawasaki et al. 2001)	
Hormonal induced	Gda-1 (gibberellic acid-induced gene)
	Asr1 (ABA and stress-induced protein)
	Osr40c 1 (ABA and salt-induced protein)
Protein destination	Subtilisin-chymotrypsin inhibitor 2, trypsin inhibtor 1
	Calcium-dependent protein kinase, nucleoside
Regulatory	lipoprotein kinase
	Calmodulin, protein phosphatase 2C homolog, elongation factor 1
	40S ribosomal protein S4, 40S ribosomal protein S7
Stress responsive genes	Glycine/serine-rich protein (grp) 1, grp 2
Unknown	5 unknown genes
Rice seedlings exposed to 150mM NaCl for 24th and 7 days (Kawasaki et al. 2001)	
Antioxidants	Glutathione-S-transferase, ascorbate peroxidase, cyt
Aquaporins	Water channel protein I and water channel protein IV
Hormonal induced	Gda-1 (gibberellic acid-induced gene)
	Osr40c1 (ABA and salt-induced protein), Osr40g2
Protein destination	Trypsin inhibtor 1, metallothionein-like protein
Unknown	3 unknown genes
Foxtail millet seedlings exposed to 250mM NaCl for 7 days (Sreenivasulu et al. 2004)	
Antioxidants	Glutha\ione peroxidase, L-ascorbate peroxidase, cyt, catalae
	Trypsin inhibitor, subtilisin-chymotrypsin inhibitor
	Kruppel-like transcription factor, argonaute protein
Regulator	Cyclophilin
Unknown	1 unknown gene

produced from salt-treated cDNA library of *Glycine max*, 1159 ESTs from *A. thaliana* and 20 ESTs from *L. esculentum*. From monocots, approximately 3331 salt-stress related ESTs were produced from *Zea mays*, 2296 from *Triticum aestivum*, 1701 from *O. sativa* and 841 from *H. vulgare*. Rapid increase of EST collections from various species of glycophytes such as soybean, tomato, barley, maize, rice and sorghum, halophyte plant *M. crystallinum* and unicellular halotolerant cyanobacterum and *Saccharomyces* will help to find the orthologs of salt-stress regulated genes, that may be common to all species (see Bohnert et al. 2001). Based on the EST collection available from salt-treated cDNA libraries, further insights into gene functions that are coupled to salt tolerance can be explored by high throughput expression profiling. Employment of high-throughput gene expression profiling based on cDNA arrays to study leaf, root, flowers and caryopsis development was shown in the recent years (Sreenivasulu et al. 2002a; 2002b; 2004). Expanding the global transcript profiling based on microarray method to compare salt tolerant and sensitive cultivars within same species under salt stress and control conditions will allow identifying significantly higher number of transcripts and various pathways related to salt-tolerant mechanisms. Recently, Kawasaki et al. (2001) made an attempt to study the large-scale gene expression profiling in salt tolerant rice variety Pokkali during high salinity treatment. Such intensive programmes are expected to yield valuable results and help to understand the complex mechanisms of salt tolerance in plants. Since salt tolerance is a multigenic trait, it requires the transfer of more than one gene preferably with the salt inducible promoters.

Conclusions

In recent years, an enormous increase in our knowledge on plant's responses to stress including salt stress has been achieved. Several genes were isolated from microorganisms and plants and transferred into crop plants to improve tolerance against salinity. Most reports state an increased salt tolerance of the respective transgenic lines. Since engineered higher accumulation of osmolytes and stress proteins also increased tolerance for water and cold

stresses, the gain in agricultural productivity with such plants would be even more dramatic. However, no scientific reports on extensive field tests were published yet. Only such trials can show which approaches are promising for further development of varieties able to meet the economical demands. In most of the reviewed experiments the foreign gene was constitutively expressed and thus is likely to cause unwanted effects. Therefore, it is highly desirable to achieve organ-specific and salt-responsive expression of the introduced genes. Especially genomics approaches could be used to identify a set of salt-inducible genes. Their promoters can be isolated and thoroughly tested for specificity. Several different such promoters are advisable to prevent gene silencing when gene pyramiding is tried as a promising technology to achieve higher tolerance levels. Genomic approaches are also highly suitable to identify whole pathways involved in salt stress response and their relationships to the whole metabolic network. At the same time general pathway regulators can be identified. However, unwanted side effects of manipulating such regulatory genes are even more likely than with single gene members of a pathway and, therefore, need intensive research and breeding efforts to use their great potential. Besides genetic engineering approaches, other more classical approaches, not reviewed here, are of no less importance. The existing genetic variability available in many crop plants and their wild relatives is increasingly and successfully used in breeding salt tolerant varieties. Thus, we are optimistic that the high expectations raised by the recent reports on more salt-tolerant plant prototypes can be met in the not too distant future.

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