Breeding for Abiotic Stress Resistance: Challenges and Opportunities

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Abstract
Recent experimentation with transgenic plants has led to increased salinity tolerance, with emphasis on the areas of ion homeostasis, osmotic regulation and antioxidant protection. A case study of the major challenges and opportunities to improve stress tolerance in plants using salinity is presented. As different abiotic stresses are inter-related (e.g. salinity and osmotic stress), our ability to improve crop performance may well be determined by combining different, apparently unrelated approaches for introducing several stress tolerance mechanisms into specific crop plants.

Introduction
Soil salinity is one of the major abiotic stresses reducing agricultural productivity (Boyer, 1982). The levels of salt inimical to plant growth affect large terrestrial areas of the world. It is estimated that more than a third of all of the irrigated land in the world is presently affected by salinity. This is exclusive of the regions classified as arid and desert lands (which comprise 25% of the total land of our planet). The loss of farmable land due to salinization is directly in conflict with the needs of the world population which is projected to increase by 1.5 billion in the next 20 years. The damaging effects of salt accumulation in agricultural soils have influenced ancient and modern civilizations. Although famine in the world nowadays is a complex problem and often not the direct result of an insufficient production of food, there is no doubt that the gains in food production provided by the "Green Revolution" have reached the ceiling. Therefore, increasing the yield of crop plants in optimal soils and in less productive lands, including salinized lands, is essential for feeding the world.

The need to produce stress tolerant crops was evident even in ancient times (Jacobsen and Adams, 1958). However, efforts to improve crop performance under environmental stresses have not been much fruitful because the fundamental mechanisms of stress tolerance in plants remain to be completely understood. Epstein *et al*. (1980) described technical and biological constraints to the problem of salinity. While there appears more success with the technical solutions to the problem, the biological solutions have been more difficult to develop. For the biological approach in raising salt tolerance to work, identification of the genetic basis of stress tolerance and using the requisite salt stress tolerance related genes or QTL (Quantitative Trail Loci) to develop varieties with enhanced salinity tolerance are a pre-requisite. The existence of salt-tolerant halophytes and differences in salt tolerance between genotypes within salt-sensitive glycophytes species clearly indicates that there is a genetic basis to salt response. While varietal differences in salt tolerance have been known since the 1930s (Epstein, 1977, 1983) and intra-specific selection for salt tolerance reported in rice (Akbar and Yabuno, 1977) and barley (Epstein *et al*., 1980), there exists still a large gap in our understanding. Flowers and Yeo (1995) reviewed the evidence for the paucity of salt-tolerant cultivars and concluded that the number was likely to be fewer than 30. Since 1993, there have been just three registrations of salt-resistant cultivars in *Crop Science* (Owen *et al*., 1994; Al-Doss and Smith, 1998; Dierig *et al*., 2001).

Two basic genetical approaches currently being utilized to improve stress tolerance include: (1) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of QTLs and subsequent marker-assisted selection and (2) generation of transgenic plants to introduce novel genes or alter expression levels of the existing genes to affect the degree of salt stress tolerance. We discuss these approaches in somewhat detail, focusing on the recent experimentation

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with transgenic plants that has led to increased salinity tolerance, with emphasis on the areas of ion homeostasis, osmotic regulation and antioxidant protection. There is an emerging body of work in the area of signaling and transcriptional control that has been recently reviewed (Hasegawa *et al,* 2000; Zhu, 2001, 2002) and thus will not be dealt with here. Finally, we will outline some of the major challenges and opportunities to improve stress tolerance in plants using salinity as a case study. As different abiotic stresses are inter-related (e.g. salinity and osmotic stress), our ability to improve crop performance may well be determined by combining different, apparently unrelated approaches for introducing several stress tolerance mechanisms into specific crop plants.

Genetics of salt tolerance

Tomato has been a valuable species to analyze the genetic basis of salinity tolerance because making successful crosses between wild and cultivated tomato plants are relatively simple. Lyon (1941) made one of the first attempts to evaluate the inheritance of salt tolerance. An interspecific cross of *Lycopersicon esculentum* and *L. pimpinellifolium* showed that the fruit yield of the hybrid was more sensitive to increasing salt (Na_2SO_4) than either parent. Other crosses of wild and cultivated tomato also suggested a complex genetics. Heterosis was apparent under saline (NaCl) conditions in the elongation of stems in hybrids of L. *esculentum* produced with three wild species (*L. cheesmanii*, *L. peruvianum*, *and L. pennellii* =*Solanum pennellii*) (Tal and Shannon, 1983). Stem elongation was a dominant trait in hybrids with *S. pennellii*, but not with *L. cheesmanii* as the parent. Total dry matter production of another F_1 hybrid between *L. esculentum* and *L. pennellii* showed hybrid vigour under saline conditions (Saranga *et al*., 1991).

Analysis of other plant species has also suggested that the genetics of salt tolerance is complex. In rice, sterility, an important factor in yield under saline conditions, is determined by at least three genes (Akbar *et al*., 1972; Akbar and Yabuno, 1977). In a genetic analysis the effects of salinity on the seedling stage and on sterility suggested both additive and dominance effects, some with high heritability (Moeljopawiro and Ikehashi, 1981; Akbar *et al*., 1986). The evidence of dominance of tolerance is also seen with pigenopea (*Cajanus cajan*), where a cross with *Atylosia albicans* (a salt-tolerant relatives of pigeonpea) produced intergeneric hybrids that were as salt tolerant as the wild parent, indicating that dry weight production was determined by a dominant genetic factor (Subbarao *et al*., 1990). There is also evidence of dominance in the salt tolerance of sorghum. Diallel analysis, based on assessing root tolerance to NaCl in salt-treated as compared with control plants, showed that there were both additive and dominance effects of NaCl (Azhar and McNeilly, 1988). Above examples clearly indicate that salinity tolerance, as in case of growth and yield, appear to be a complex trait.

Early attempts to evaluate the genetic basis of stress tolerance in plants were restricted to simple genetic models. However with the development of molecular markers, evaluating the inheritance of salinity tolerance became a more tractable problem since specific QTLs could be identified. Through the development of molecular markers, it has become possible not only to determine the genetic basis of the trait, but to also map specific chromosomal segments or QTL and determine the relative contribution of each QTL to the variance observed for the trait. Genomic maps have been constructed in various crops to exploit genetic diversity (Thormann *et al.,* 1994), tag qualitative and quantitative traits (Butruille *et al.,* 1999) and analyze the stability of detected QTL across different environments (Hittalmani *et al.,* 2002). Stable and consistent QTLs provide an excellent opportunity to improve the efficiency of selection, especially for traits controlled by multiple genes and highly influenced by the environment, as is the case for salinity (Dudley 1993). The efficiency of marker assisted selection (MAS) is dependent on a number of factors such as the distance of observed QTL from marker loci (Dudley 1993) and the proportion of the total additive variance explained by the QTL (Lande and Thompson 1990). There is considerable evidence to support the view that salt tolerance and its sub-traits are determined by multiple QTLs. In an intergeneric cross of tomato, several QTLs were found associated with fruit yield in plants growing under saline conditions (Breto *et al*., 1994), although some of the QTL identified were later shown to be dependent on the parentage of the cross (Monforte *et al*., 1997*a*). Some QTL associated with specific aspects of fruit yield were found regardless of whether the plants were grown with or without salt; others were detected only under saline or under non-saline conditions (Monforte *et al*., 1997*b*). Other crosses have also identified both stress- (salt and cold) specific and stress-non-specific QTLs. The stress-non-specific QTL generally exhibited larger individual effects and accounted for a greater portion of the total phenotypic variation under each condition than the stress-specific QTL (Foolad *et al*., 1999). As for the

QTL identified for fruit yield, QTL associated with germination depend upon the conditions under which germination is assessed (Foolad *et al*., 1999). A similar situation exists for citrus, where about half of the potential QTL identified depended on the presence or absence of salinity (Tozlu *et al*., 1999*a*), and in rice (Gong *et al*., 1999, 2001) where less than 10% of the QTL were detected both in the presence and absence of salt. Clearly, the major determinants of yield vary with the environmental conditions and quantitative traits typically exhibit a large environment(x)genotype interaction.

The use of tomato has also been important in establishing that QTL associated with tolerance vary with the stage of plant development. The QTL associated with tolerance at germination (Foolad *et al*., 1997, 1998) and vegetative growth (Foolad and Chen, 1999; Foolad *et al*., 2001) are shown to differ (Foolad, 1999). Such differences are not restricted to tomato; same have been demonstrated in *Arabidopsis* (Quesada *et al*., 2002) and barley (Mano and Takeda, 1997). QTL associated with aspects of ion transport have also been reported in citrus (Tozlu *et al*., 1999*b*) and rice (Koyama *et al*., 2001).

Salt tolerance using transgenic approach

Physiologically, salinity (a) imposes water-deficit that results from the relatively high solute concentrations in the soil, (b) causes ion-specific stresses resulting from altered K^+/Na^+ ratios and (c) leads to build up in Na^+ and Cl^- concentrations that are detrimental to plants. Here, we discuss following three key processes that contribute to salt tolerance at the cellular level: (a) the establishment of cellular ion homeostasis, (b) the synthesis of compatible solutes for osmotic adjustment, and (c) the increased ability of the cells to neutralize reactive oxygen species generated during the stress response.

Ion homeostasis: Although Na^+ is required in some plants, particularly halophytes (Glenn *et al.,* 1999), a high NaCl concentration is a toxic factor for plant growth. The alteration of ion ratios in plants is due to the influx of Na^+ through pathways that function in the acquisition of K^+ (Blumwald *et al.,* 2000). The sensitivity to salt of cytosolic enzymes is similar in both glycophytes and halophytes, indicating that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in high salt (Glenn *et al.,* 1999). Strategies that plants could use in order to maintain a high K^+/Na^+ ratio in the cytosol include: (i) extrusion of Na^+ ions out of the cell and (ii) vacuolar compartmentation of Na^+ ions. Under typical physiological conditions, plants maintain a high cytosolic K^+/Na^+ ratio. Given the negative membrane potential difference at the plasma membrane (-140 mV) (Higinbotham, 1973) (Fig. 1), a rise in extracellular Na^+ concentration will establish a large electrochemical gradient favoring the passive transport of Na^+ into the cells. Na^+ extrusion from plant cells is powered by the operation of the plasma membrane H^+-ATPase generating an electrochemical H^+ gradient that allows plasma membrane Na^+/H^+ antiporters to couple the passive movement of H^+ inside the cells, along its electrochemical potential, to the active extrusion of Na^+ (Blumwald *et al.,* 2000). Recently, *AtSOS1* from *Arabidopsis thaliana* has been shown to encode a plasma membrane Na^+/H^+ antiport with significant sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria and fungi (Shi *et al.,* 2000). The overexpression of *SOS1* improved the salt tolerance of *Arabidopsis*, demonstrating that improved salt tolerance can be attained by limiting Na^+ accumulation in plant cells (Shi *et al.,* 2003). The compartmentation of Na^+ ions into vacuoles also provides an efficient mechanism to avert the toxic effects of Na^+ in the cytosol. The transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter that is driven by the electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-PPiase (Blumwald, 1987). The overexpression of a *AtNHX1,* a vacuolar Na^+/H^+ antiporter from *Arabidopsis,* in *Arabidopsis* resulted in transgenic plants that were able to grow in high salt concentrations (Apse *et al.,* 1999). The paramount role of Na^+ compartmentation in plant salt tolerance has been further demonstrated in transgenic tomato and Canola plants overexpressing AtNHX1 (Zhang and Blumwald, 2001, Zhang *et al.,* 2001). Additional evidence supporting the role of vacuolar transport in salt tolerance has been provided by *A. thaliana* plants overexpressing a vacuolar H^+-PPiase (Gaxiola *et al.,* 2001). Transgenic plants overexpressing *AVP1,* coding for the vacuolar H^+-pyrophosphatase, displayed enhanced salt tolerance that was correlated with the increased ion content of the plants. These results suggest that the enhanced vacuolar H^+-pumping in the transgenic plants provided additional driving force for vacuolar sodium accumulation via the vacuolar Na^+/H^+ antiporter.

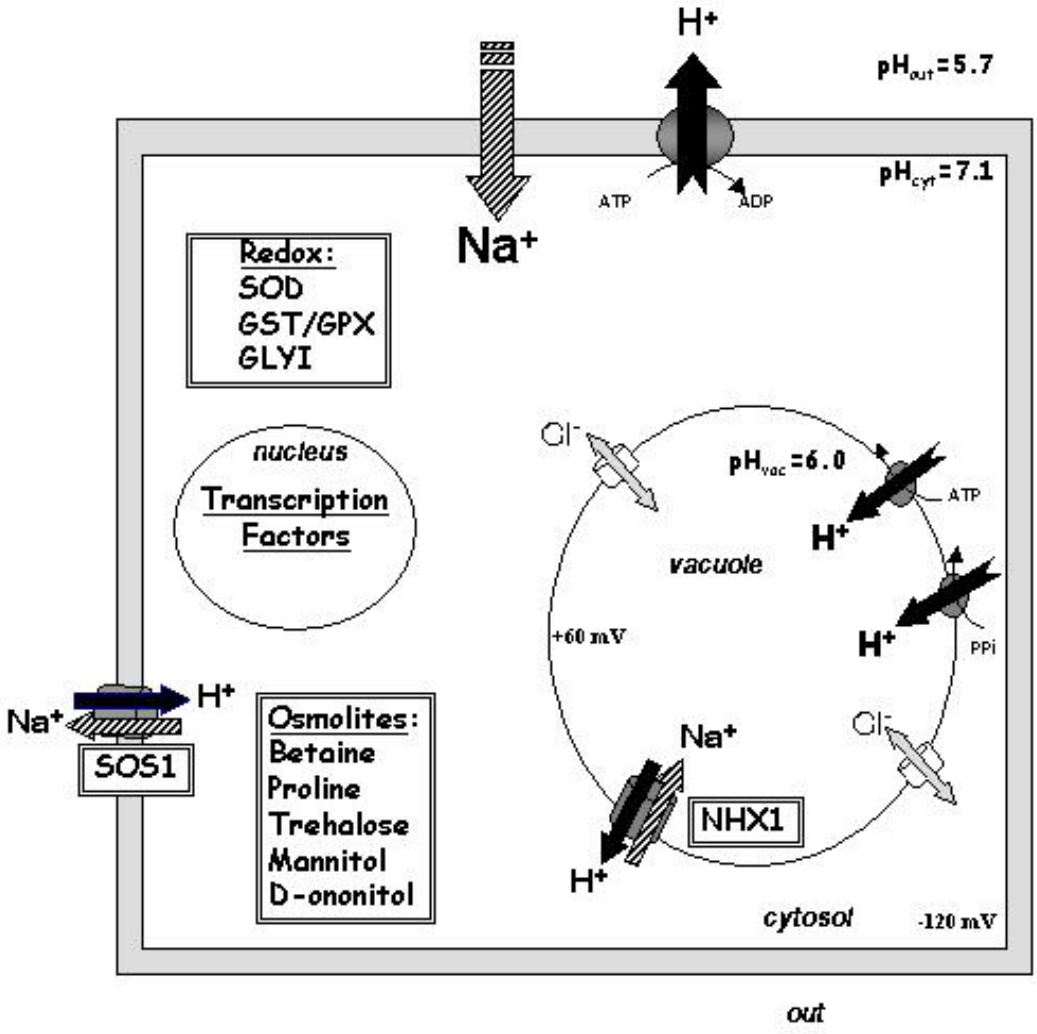

Figure 1. Schematic representation of primary and secondary transport in the plant cells. Electrogenic H^+ transport (H^+-ATPase in the plasma membrane and vacuolar membrane, H^+-PPiase in the vacuolar membrane) generates gradients of pH and electrical potential difference across the cell and vacuolar membranes. Na^+ ions enter the cell and can be translocated out of the cell or into the vacuole by the action of a plasma membrane Na^+/H^+ antiporter (SOS1) or a vacuolar Na^+/H^+ antiporter (NHX1), respectively.

Synthesis of compatible solutes**:** The cellular response of salt tolerant organisms to both long- and short-term salinity stress includes the synthesis and accumulation of a class of osmoprotective compounds known as compatible solutes. These relatively small, organic osmolytes include amino acids and derivatives, polyols and sugars, methylamines etc. The osmolytes are consider to 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 and protein integrity, which is perturbed in the face of soil solutions containing higher amounts of NaCl and the consequent loss of water from the cell. The accumulation of osmotically active compounds in the cytosol increases the osmotic potential to provide a balance between the apoplastic solution, which itself becomes more concentrated with Na^+ and Cl^- ions, and the vacuolar lumen, which in halophytes can accumulate up to 1 M Na^+ (and Cl^-). For a short-term stress, this may provide the cells with the ability to prevent water loss. However, for continued growth under salinity stress, an osmotic gradient (towards the cytosol) must be kept in order to maintain turgor, water uptake and facilitate cell expansion.

The enhancement of proline and glycinebetaine synthesis in target plants has received much attention (Rontein *et al.,* 2002). Two themes have emerged from the results of these efforts: (i) there are metabolic limitations on the absolute levels of the target osmolyte that can be accumulated and (ii) the degree to which the transformed plants are able to tolerate salinity stress is not necessarily correlative with the amounts of the osmoprotectants attained. The metabolic limitations on increasing the concentration of a

given osmoprotectant is well illustrated with both proline and glycinebetaine. Initial strategies aimed at engineering higher concentrations of proline began with the over-expression of genes encoding the biosynthetic enzymes pyrroline-5-carboxylate (P5C) synthase (P5CS) and P5C reductase (P5CR) that catalyze the two steps between the substrate, glutamic acid and the product, proline. P5CS over-expression in tobacco dramatically elevated free proline in transgenic tobacco (Kishor *et al.,* 1995). However, the regulation of free proline does not appear to be straight-forward. Proline catabolism, via proline dehydrogenase (ProDH), is upregulated by free proline and there exists a strong evidence that free proline inhibits P5CS (Roosens *et al.,* 1999). Further, a two-fold increase in free proline was achieved in tobacco plants transformed with a *P5CS* modified by site-directed mutagenesis (Hong *et al.,* 2000). This modification alleviated the feedback inhibition by proline on the P5CS activity and resulted in an improved germination and growth of seedlings under salt stress. Free cellular proline levels are also transcriptionally and translationally controlled. P5CR promoter analysis revealed that P5CR transcripts have reduced translational initiation. A 92 bp segment of the 5'UTR of P5CR was sufficient to provide increased mRNA stability and translational inhibition under salt stress to the GUS reporter gene that was been ligated at 3' end to this small region (Hua *et al.,* 2001). These results highlighted the complex regulation of P5CR during stress and emphasized the importance of stability and translation of P5CR mRNA during salt stress. An alternative approach to attain significant free proline levels, where antisense cDNA transformation was used to decrease ProDH expression, was utilized (Nanjo *et al.* 1999). Levels of proline in the transgenic *Arabidopsis* were twice (100 μg/ g fresh weight) that of control plants grown in the absence of stress, and three times higher (600 μg/ g fresh weight) than in control plants grown under stress. The high levels of proline were correlated with an improvement in tolerance to salinity, albeit for a short duration exposure to 600 mM NaCl.

There has been considerably more experimentation directed at the engineering of glycinebetaine synthesis than for any other compatible solute. Unlike proline, glycinebetaine degradation is not significant in plants (Nuccio *et al.,* 2000), but the problems of metabolic fluxes, compounded with the compartmentation of the substrate and product pools, has made the engineering of appreciable levels of glycinebetaine problematic. In plants that are naturally glycinebetaine accumulators (spinach and sugarbeet), synthesis of this compound occurs in the chloroplast, with two oxidation reactions from choline to glycinebetaine. The first oxidation to betaine aldehyde is catalyzed by choline monooxygenase (CMO), an iron-sulfur enzyme. Betaine aldehyde oxidation to glycinebetaine is catalyzed by betaine aldehyde dehydrogenase (BADH), a non-specific soluble aldehyde dehydrogenase (Rathinasabapathi, 2000). In *E. coli*, these reactions are cytosolic; in this species the first reaction is catalyzed by the protein encoded by the *betA* locus choline dehydrogenase (CDH), which is an NAD^+-dependent enzyme, and BADH in E.coli, is encoded by the *betB* locus. 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). The codA gene of *A. globiformis* offers an attractive alternative to the engineering of glycinebetaine synthesis as it necessitates only a single gene transformation event. This strategy was employed for engineering glycinebetaine synthesis in *Arabidopsis* (Hayashi *et a.,* 1997). The 35S promoter driven construct for transformation included the transit peptide for the small subunit of Rubisco so that the COD protein would be targeted to the chloroplast. Improved salinity tolerance was obtained in transgenic *Arabidopsis* that accumulated, as a result of the transformation, 1 μmol/ g fresh weight glycinebetaine. The same construct was used by for transformation of *Brassica juncea* (Prasad *et al.,* 2000) and tolerance to salinity during germination and seedling establishment was improved markedly in the transgenic lines. COX from *Arthrobacter panescens*, which is homologous to the *A. globiformis* COD, was used to transform *Arabidopsis*, *Brassica napus* and tobacco (Huang *et al.,* 2000). This set of experiments differs from those above in that the COX protein was directed to the cytoplasm and not to the chloroplast. Improvements in tolerance to salinity and drought and freezing were observed in some transgenics from all three species, but the tolerance was variable. The levels of glycinebetaine in the transgenic plants were not significantly higher than those of wild type plants, but increased significantly with the exogenous supply of choline to plants, suggesting that the supply of choline is a significant constraint on the synthesis of glycinebetaine (Huang *et al.,* 2000).

Two important issues emerge from the results of the above discussion. The first is that the concentrations of glycinebetaine in the transgenic plants were much lower than the concentrations noted in natural accumulators. Despite the fact that these levels are not high enough to be osmotically significant, a moderate (and significant) increase in tolerance to salinity and other stresses was conferred. This raises

the possibility that the protection offered by glycinebetaine is not only osmotic, which is a point raised by several of the above groups. Same explanation was also offered by Bonhert and Shen (1999). Compatible solutes, including mannitol may also function as scavengers of oxygen radicals, which may be supported by the results of Alia *et al.* (1999), where the protection to photosystem II in plants expressing codA was observed. An alternative possibility, not necessarily exclusive of the first, is that the increased level of peroxide generated by the COD/COX oxidation of choline causes an upregulation of ascorbate peroxidase and catalase (Holmstrom *et al.,* 2000) which may also improve the tolerance to salinity stress (Rontein *et al.,* 2002). The second issue is that the level of glycinebetaine production in the transgenics is limited by choline. Because betaine synthesis takes place in the chloroplast, the free choline pool may not reflect its availability to the chloroplast, which may be limited in this compartment by the activity and/ or abundance of choline transporters. However, a dramatic increase in glycinebetaine levels (to 580 μmol/ g dry weight in *Arabidopsis*) was shown in the transgenic plants when they were supplemented with choline in the growth medium (Huang *et al.,* 2000). This limitation was not explored in the transgenic tobacco expressing *E. coli* enzymes CDH and BADH in the cytoplasm (Holmstrom *et al.,* 2000). Although these transgenic plants demonstrated an improved tolerance to salinity, glycinebetaine levels were on the order of those mentioned above. Sakamoto and Murata (2000) also asserted that despite the similarities in tolerance exhibited by transgenic plants engineered to synthesize betaine in either the chloroplast or cytoplasm, the site of synthesis of betaine may play a role in the degree of tolerance shown. Indeed, if the betaine present in these plants is localized primarily in the chloroplast, it may be present at significant concentrations (50 mM) (Hayashi *et al.,* 1997). However, Sakamoto and Murata (2000) downplayed the limitation of the metabolic pool of choline on the levels of glycinebetaine obtained in the engineered plants, by suggesting that the choline oxidizing activity may be the limiting factor. This argument seems to be supported by Huang *et al.* (2000) who found that the levels of glycinebetaine correlated with the levels of COX activity measured in each plant. The increase in glycinebetaine with exogenous choline argues against this notion. Stronger evidence for the limitations of choline metabolism have been presented by McNeil *et al.* (2001). By over-expressing spinach phosphoethanolamine N-methyltransferase (PEAMT), which catalyzes the three methylation reactions required for the conversion of phosphoethanolamine to phosphocholine, up to a 50-fold increase in free choline was obtained. This led to an increase in glycinebetaine levels (+60%) in plants that were expressing spinach CMO and BADH in the chloroplast. Further, the addition of ethanolamine to the plant growth medium caused an increased choline and glycinebetaine levels, showing that the metabolic flux through this pathway is also limited by the supply of ethanolamine. As PEAMT is itself inhibited by phosphocholine, further engineering efforts need to include (a) the modification of PEAMT to remove this inhibition (McNeil *et al.,* 2001), (b) increasing the supply of ethanolamine by overexpression of serine decarboxylase and (c) resolving the compartmentation problem of choline supply and choline oxidation, either by use of choline oxidation in the cytoplasm or by finding the appropriate transporters to improve choline supply to the chloroplast (Rontein *et al.,* 2002).

Finally, as the compatible solutes are non-toxic, the interchangeability of these compounds between species has held much interest (Table 1). The recent examples include the engineering of (a) ectoine synthesis with enzymes from the halophylic bacterium *Halomonas elongata* in plants (Ono *et al.,* 1999; Nakayama *et al.,* 2000) and (b) trehalose synthesis which occurs in bacteria, yeast and in extremely desiccation-tolerant plants (Goddijn and van Dun, 1999) into potato (Yeo *et al.,* 2000) and rice (Garg *et al.,* 2002). An intriguing report on the improved tolerance to salinity in tobacco expressing yeast invertase in the apoplast highlights the potential of manipulating sucrose metabolism (Fukushima *e al.,* 2001). The authors reported improved salt tolerance of transgenic tobacco plants expressing an yeast invertase in their apoplastic space, and concluded that the changes in sucrose metabolism in the transgenic plants protected the photosynthetic apparatus under salt stress. The overexpression of polyols, such as mannitol (Tarczynski *et al.,* 1993) and D-ononitol (Sheveleva *et al.,* 1997) have been shown to contribute to enhanced drought and salt tolerance in transgenic tobacco plants.

TABLE 1. Salt tolerance in transgenic plants expressing genes involved in osmolyte biosynthesis, ion transporters, redox proteins and transcription factors.

Gene	Gene Product	Source	Cellular role(s)	Target plant	Parameter studied	Reference
Osmolytes and Compatible solutes						
betA	Choline dehydrogenase	E. coli	Glycinebetaine	Tobacco	Dry weight	Lilius *et al.* 1996 Holmstrom *et al.* (2000)
BADH	Betaine dehydrogenase	spinach	Glycinebetaine	Tobacco		Liang *et al.* 1997
EctA, ectB, ectC	L-2,4-diaminobutyric acid acetyltransferase, L-2,4-diaminobutyric acid trans-aminase, L-ectoine synthase.	Halomonas elongata	Ectoyne	Tobacco	Salinity tolerance	Nakayama *et al.* 2000
OtsA OtsB	Trehalose-6-P synthase Trehalose-6-P phosphatase	E. coli	Trehalose	Tobacco Rice	Increased biomass, morphogenesis Growth	Pilon-Smits *et al.* 1998 Garg *et al.* 2002.
TPS1	Trehalose-6-phosphate synthase	S. cerevisiae	Trehalose	Tobacco	Improved drought tolerance	Romero *et al* 1997
P5CS	Δ^1-Pyrroline-5-carboxylate synthase	V. aconitifolia	Proline	Tobacco	Increased proline; plant growth	Kishor *et al.* 1995
ProDH	Proline dehydrogenase	Arabidopsis thaliana	Proline	Arabidopsis	Inflorescence lodging in response to NaCl stress	Nanjo *et al.* 1999
IMT1	Myo-inositol-*O*-methyl transferase	Messembryanthemum chrystallinum	D-Ononitol	Tobacco	Seed germination	Vernon *et al.* 1993; Sheveleva *et al.* 1997
COD1; COX	Choline oxidase	Arthobacter globiformis; Arthobacter panescens	Glycinebetaine	Arabidopsis Rice Brassica	Seed germination; plant growth	Hayashi *et al.* 1997 Alia *et al.* 1998 Sakamoto *et al.* 1998 Huang *et al.* 2000 Prasad *et al.* 2000
HAL3	FMN-binding protein	S. cerevisiae	K^+/Na^+ homeostasis	Arabidopsis	Seedlings	Espinosa-Ruiz *et al.*1999
Ion Transporters and Ion Homeostasis						
AtNHX1	Vacuolar Na^+/H^+ antiporter	A. thaliana	Na^+ vacuolar sequestration	Tomato Arabidopsis B. napus	Biomass, fruit and oil production	Apse *et al.* 1999 Zhang and Blumwald, 2001; Zhang *et al.* 2001
AtSOS1	Plasma	A. thaliana	Na^+ extrusion	Arabidopsis	Biomass	Shi *et al.*

Gene	Gene Product	Source	Cellular role(s)	Target plant	Parameter studied	Reference
	membrane Na^+/H^+ antiporter					2003
AVP1	Vacuolar H^+-pyrophosphatase	A. thaliana	Vacuolar acidification	Arabidopsis	Biomass	Gaxiola *et al*. 2001
HAL1	K^+/Na^+ transport regulation	S. cerevisiae	K^+/Na^+ homeostasis	Tomato melon *Arabidopsis*	Sustain of K^+/Na^+ ratio; plant growth	Bordas *et al*. 1997 Gisbert *et al*. 2000 Yang *et al*. 2001
Redox proteins						
MnSOD	Superoxide dismutase	S. cerevisiae	Reduction of O_2 content	Rice		Tanaka *et al*. 1999
GlyI	Glyoxylase	B. juncea	S-D-Lactoylglutathi-one	Tobacco	Chlorophyll content of detached leaves	Veena *et al.* 1999
TPX2	Peroxidase	N. tabaccum	Change cell wall properties	Tobacco	Germination ; water retention in seed walls	Amaya *et al*. 1999
GST GPX	Glutathione S-transferase Glutathione peroxidase	N. tabaccum N. tabaccum	ROS scavenging	Tobacco	Germination and growth	Roxas *et al*. 2000
Transcription and Signal Transduction Factors						
DREBIA	Transcription factor	A. thaliana	Improved gene expression	Arabidopsis	Plant growth and survival rate	Kasuga *et al*. 1999
Cnb1	Calcineurin	S. cerevisiae	Improved Ca^{2+} signaling	Tobacco	Plant growth	Pardo *et al*. 1998
OsCDPK7	Protein kinase	O. sativa	Improve gene expression	Rice	Wilty phenotype	Saijo *et al*. 2000
Others						
DnaK	Heat shock protein	A. halophytica	Protein stabilization	Tobacco	CO_2 fixation, Na^+ content	Sugino *et al.* 1999
Apo-Inv	Apoplastic yeast-derived invertase	S. cerevisiae	Sucrose synthesis	Tobacco	Photosynthetic activity and osmotic pressure	Fukushima *et al.* 2001

Antioxidant protection: An important aspect of salinity stress in plants is the stress-induced production of reactive oxygen species (ROS) including superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-). ROS are a product of altered chloroplast and mitochondria metabolism during stress. These ROS cause oxidative damage to different cellular components including membrane lipids, protein and nucleic acids (Halliwell and Gutteridge, 1986). The alleviation of this oxidative damage could provide enhanced plant resistance to salt stress. Plants use low molecular mass antioxidants such as ascorbic acid and reduced glutathione and employ a diverse array of enzymes such as superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione S-transferases (GST) and glutathione peroxidases (GPX) to scavenge ROS. Transgenic rice over-expressing yeast mitochondrial Mn-dependent SOD displayed enhanced salt tolerance (Tanaka *et al.,* 1999). The overexpression of a cell wall peroxidase in tobacco plants improved germination under osmotic stress (Amaya *et al.,* 1999). Transgenic tobacco plants over-expressing both GST and GPX displayed improved seed germination and seedling growth under stress (Roxas *et al.,* 1997). Subsequent studies (Roxas *et al.,* 2000) demonstrated that in addition to increased GST/GPX activities, the transgenic seedlings contained higher levels of glutathione and ascorbate than wild-type seedlings, showed higher levels of monodehydroascorbate reductase activity and the glutathione pools were more oxidized. These results would indicate that the increased glutathione-dependent peroxidase scavenging activity and the associated changes in glutathione

and ascorbate metabolism led to reduced oxidative damage in the transgenic plants and contributed to their increased salt tolerance.

Assessment of salt tolerance in transgenic plants

The assessment of salt tolerance in transgenic experiments as described above has been mostly carried out using limited number of seedlings/ mature plants in laboratory experiments. However, the level of salt tolerance of crops ultimately needs to be evaluated as yield from a producer's fields. The evaluation of field performance under saline stress conditions is difficult because of the variability of salt levels in field-conditions (Richards, 1983; Daniells *et al*., 2001) and the potential for interactions with other environmental factors, including soil fertility, temperature, light intensity and transpirational water loss. Some work also aims at the prediction of field performance carried out in trial plots, or using a solution-based method where the salinity of the medium can be readily adjusted to required values (Francois and Maas, 1994). This type of experiment often precludes measuring yield through lack of space and estimates of tolerance obtained from such experiments are often not borne out by the response of plants in the field (Rowland *et al*., 1989; Daniells *et al*., 2001). Evaluating tolerance is made more complex by variation in sensitivity to salt during the life cycle. For example, it is known that grain yield in rice is much more depressed by salt than is vegetative growth (Khatun and Flowers, 1995): germination is relatively salt resistant. In tomato, tolerance at germination is not correlated with the ability to grow under salt stress: both are controlled by different mechanisms (Foolad and Lin, 1997), although some genotypes display similar tolerance at germination and during vegetative growth (Foolad and Chen, 1999).

It thus needs to be recognized that the assessment of stress tolerance in the laboratory often has little correlation to tolerance in the field. Although there have been many successes in developing stress-tolerant transgenics in model plants such as tobacco, *Arabidopsis* or rice (Grover *et al,*. 2003), there is urgent need to test these successes in crops. Rice has the advantage that it is both the model monocot and an important crop. However, same is not the case when transgenes are tested with tobacco or Arabidopsis (reviewed in Grover *et al.,* 2003; Flowers, 2004). This brings a number of technical and financial challenges associated with transforming many of the crop plants, particularly the monocots. First, transformation of any monocot other than rice is still not routine and to develop a series of independent homozygous T2 lines is costly, both in money and time. Second, the stress tolerance screens will need to include a field component since many of the stress tolerance assays used by basic researchers involve using rich nutrient media which include sucrose. This type of screen is unlikely to have relationship to field performance. Finally, since saline soils are often complex and may include NaCl, $CaCl_2$, $CaSO_4$ and Na_2SO_4, plants that show particular promise will eventually have to be tested in all of these environments.

Challenges and Opportunities

Conventional breeding programs for raising salt tolerant genotypes have meet with limited success. This lack of success is in part because breeders prefer to evaluate their genetic material in ideal conditions. This issue is getting more urgent to deal with the growing interest of seed companies in making salt tolerant crops. Because of the fact that producers in general prefer to avoid using land that has reduced yields due to salinity, and plant breeding companies in general tend to develop varieties under conditions which are optimal, the efforts necessary to produce new salt-tolerant cultivars remain inconsequential. From a business perspective, in order for plant breeding companies to invest in the development of new varieties with enhanced stress tolerance, there will always be the question raised as to whether the investment in the development of these cultivars is worth the effort. There is no benefit in developing salinity tolerant plants unless there are the economic drivers which will allow the plant to be competitively productive with non-saline tolerant plants growing on uncompromised soil. The viewpoints of basic researchers might differ—for the researchers the actual though small increase in salt tolerance is worth the efforts.

In evaluating the possibility of improving stress tolerance in plants, there are a number of considerations that we propose that the research community should consider including:

While it has been recognized by many researchers that there are dramatic changes in gene expression associated with all types of stresses, the promoters that are most commonly used for transgene introductions are primarily constitutively expressed, including the CaMV35S promoter, ubiquitin, and actin promoters (Grover *et al,*. 2003). Recent studies have noted that the over-expression of specific stress

induced genes under the control of stress induced or tissue specific promoters often display a better phenotype than the same genes expressed under a constitutive promoter (Zhu *et al.,* 1998; Kasuga *et al.,* 1999).

Second, while there have been a number of successes in the production of abiotic stress-tolerant plants using tobacco or *Arabidopsis* using specific genes, there is a clear need to begin to introduce these tolerance genes into crop plants. Moreover, even though researchers tend to focus on a few basic plant systems, with *Arabidopsis*, tobacco and rice being the major species of choice, there has been no attempt to choose specific genetic backgrounds.

Third, it is likely that the effectiveness of a specific transgene will be based on the specific genetic environment into which it is transformed. One component of this is the well known phenomena of "position effect', however in addition, the ability of a transgene to work may well be determined by the overall genetic background, independent from the chromosomal location of the transgene, referred to as "Transgene Combining Ability" (TCA).

Finally, we also need to establish better comparative systems. At the same time, we need to look at rational concepts for combining genes, just as the disease resistance researchers are now doing with gene stacking. For example, the over-expression of AtSOS1 in meristems (non-vacuolated cells) and AtNHX1 (for vacuolar Na^+ accumulation), together with the overproduction of compatible solutes would provide not only the ability of using NaCl as an osmoticum during vegetative growth but also would provide the seedlings with the ability to reduce Na^+ toxicity during early growth and seedling establishment. Further wherever applicable, genes for protection against oxidative stress must be combined, particularly in actively photosynthesizing cells that are prone to more chloroplast damage due to ROS.

While progress in improving stress tolerance has been slow, there are a number of opportunities and reasons for optimism. Over the last ten years there has been the development of a number of the functional tools that can allow us to dissect many of the fundamental questions associated with stress tolerance. These include: (a) the development of molecular markers for gene mapping and the construction of associated maps, (b) the development of EST libraries, (c) the complete sequencing of plant genomes including *Arabidopsis*, rice and maize, (d) the production of T-DNA or transposon tagged mutagenic populations of *Arabidopsis* and (e) the development of a number of forward genetics tools that can be used in gene function analysis such as TILLING (Colbert *et al.,* 2001). Thus, we need to focus on looking at the comparative effects and interaction of specific transgenes within a defined genetic background and determine the efficacy of these approaches in the field.

Summary

Over the last 50 years, many researchers have argued for the development of salt-tolerant crops from true halophytes. Although halophytes are present in a wide diversity of plant forms, to date very few halophytic crops can compete effectively with glycophytic crops (Glenn *et al.,* 1999). Moreover, research on the physiology of tolerance suggests that the overall trait is determined by a number of sub-traits any of which might, in turn, be determined by any number of genes. We believe that by comparing different genes and genetic combinations, researchers will be able to advance the field more quickly, and develop the stress tolerant germplasm.

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