Engineering salt tolerance in plants

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Recent progress has been made in the identification and characterization of the mechanisms that allow plants to tolerate high salt concentrations. The understanding of metabolic fluxes and the main constraints for the production of compatible solutes (i.e. feedback inhibition and the limitation of substrate supply) open up the possibility of genetically engineering entire pathways that could lead to the production of osmoprotectants. This, together with the identification of the different sodium transporters (in particular vacuolar and plasma membrane Na^+/H^+ antiporters) that could provide the needed ion homeostasis during salt stress, opens the possibility of engineering crop plants with improved salt tolerance.

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Abbreviations

BADH	betaine aldehyde dehydrogenase
CDH	choline dehydrogenase
COD	choline oxidase
CMO	choline monooxygenase
GST	glutathione *S*-transferase
GPX	glutathione peroxidase
H^+-PP_iase	H^+-pyrophosphatase
P5CS	Δ^1-pyrroline-5-carboxylate synthase
P5CR	Δ^1-pyrroline-5-carboxylate reductase
PEAMT	phosphoethanolamine *N*-methyltransferase
ROS	reactive oxygen species

Introduction

Salinity imposes two stresses on plant tissues: a water-deficit that results from the relatively high solute concentrations in the soil, and ion-specific stresses resulting from altered K^+/Na^+ ratios and Na^+ and Cl^- concentrations that are inimical to plants. As salinity stress is a continuing and increasingly deleterious obstacle to the growth and yield of crop plants, owing to irrigation practices and increasing demands on fresh water supply, the engineering of salt-tolerant crop plants has been a long-held and intensively sought objective. Breeding efforts using salt-tolerant relatives of crop plants has had only limited success, although the mapping of quantitative trait loci (QTL) in tomato [1] and rice [2], and methods such as somatic hybridization [3] hold promise as a combination of conventional breeding and a molecular approach. The availability of genome sequence information and the utility of yeast as a model system for functional testing (and a source of genes) have made the identification of desirable genes much faster. Traditional mutagenesis continues to be a fruitful source of discovery, however, as exemplified by the identification of two loci in tomato that appear to be important for salt tolerance [4••]. This review focuses on the experimentation with transgenic plants that has led to increased salinity tolerance. We focus on the areas of osmotic regulation, antioxidant protection and ion homeostasis. There is an emerging body of work in the area of signaling and transcriptional control that has been recently reviewed [5–8] and owing to space constraints will not be dealt with here.

Synthesis of compatible solutes

The cellular response of salt-tolerant organisms to salinity stress (both long-term and short-term) includes the synthesis and accumulation of a class of osmoprotective compounds known as compatible solutes. These are relatively small, non-toxic compounds that can stabilize proteins and cellular structures and can increase the osmotic pressure of the cell [9]. 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 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 to maintain turgor, water uptake, and to facilitate cell expansion.

Because compatible solutes are non-toxic, the ability to interchange these compounds between species has held much interest. The most recent examples include the engineering of ectoine synthesis (with enzymes from the halophilic bacterium *Halomonas elongata*) in plants [10] and trehalose synthesis (which occurs in bacteria, yeast and in extremely desiccation-tolerant plants) [11], which has been installed in potato [12]. An intriguing report on the improved tolerance to salinity of tobacco expressing yeast invertase in the apoplast, highlights the potential of manipulating sucrose metabolism [13•]. The enhancement of proline and glycine betaine synthesis in target plants has received more attention [14••]. Two themes have emerged from the results of these combined efforts. First, there are metabolic limitations on the absolute levels of the target osmolyte that can be accumulated. Second, the degree to which transformed plants are able to tolerate salinity stress is not necessarily correlative with the amounts of osmoprotectants attained. The metabolic limitations on increasing the concentration of a given osmoprotectant is well illustrated with both proline and glycine betaine. Initial strategies aimed at engineering higher concentrations of proline began with the overexpression of genes encoding the biosynthetic enzymes Δ^1-pyrroline-5-carboxylate synthase (P5CS) and Δ^1-pyrroline-5-carboxylate

reductase (P5CR), which catalyze the two steps between the substrate, glutamic acid, and the product, proline. P5CS overexpression in tobacco dramatically elevated free proline in transgenic plants [15]; however, the regulation of free proline is not straightforward. Proline catabolism, via proline dehydrogenase (ProDH), is upregulated by free proline, and there is strong evidence for the inhibition of P5CS by free proline [16]. Recently, a twofold increase in free proline was achieved in tobacco plants transformed with a P5CS modified by site-directed mutagenesis [17•]. 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 mRNA transcripts have reduced translational initiation in the presence of elevated levels of proline. A 92 base pair (bp) segment of the 5′ untranslated region of P5CR was sufficient to provide increased mRNA stability and translational inhibition under salt stress to the GUS reporter fused 3′ to this small region [18••]. An alternative approach to attain significant free proline levels was utilized by Nanjo *et al.* [19], in which antisense cDNA transformation was used to decrease ProDH expression. Levels of proline in transgenic *Arabidopsis thaliana* 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 glycine betaine synthesis than for any other compatible solute. Unlike proline, glycine betaine degradation is not as significant in plants [20], but the problems of metabolic fluxes, compounded with the compartmentation of the substrate and product pools, has made the engineering of appreciable levels of glycine betaine problematic. In plants that are natural accumulators (e.g. spinach and sugarbeet), glycine betaine synthesis occurs in the chloroplast with two oxidation reactions from choline to glycine betaine. The first oxidation to betaine aldehyde is catalyzed by choline monooxygenase (CMO), an iron–sulfur enzyme. Betaine aldehyde oxidation to glycine betaine is then catalyzed by betaine aldehyde dehydrogenase (BADH), a non-specific soluble aldehyde dehydrogenase [21]. In *Escherichia coli*, these reactions are cytosolic, but the first reaction is catalyzed by the protein encoded by the *betA* locus, choline dehydrogenase (CDH). Like BADH, CDH is an NAD^+-dependent enzyme; in *E. coli*, BADH is encoded by the *betB* locus. However, in some microorganisms, like *Arthrobacter globiformis*, the two oxidation steps are catalyzed by one enzyme, choline oxidase (COD), which is encoded by the *codA* locus [22]. The *codA* gene of *A. globiformis* offers an attractive alternative to the engineering of glycine betaine synthesis using the plant enzymes, as it necessitates only a single gene transformation event. This strategy was employed by Hayashi *et al.* [23] for engineering glycine betaine synthesis in *A. thaliana*. The 35S promoter driven construct for transformation included the transit peptide for the small subunit of Rubisco so that COD 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 glycine betaine. The same construct was used by Prasad *et al.* [24] for the transformation of *Brassica juncea*. Tolerance to salinity during germination and seedling establishment was improved markedly in the transgenic lines. Huang *et al.* [25•] used COX from *Arthrobacter panescens*, which is homologous to the *A. globiformis* COD, to transform *Arabidopsis*, *Brassica napus*, and tobacco. 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, drought and freezing were observed in some transgenics from all three species, but the tolerance was variable among species [25•].

There are two commonalities in the results from these groups. The first is that the concentrations of glycine betaine in the transgenic plants were much lower than the concentrations seen 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 glycine betaine is not only osmotic, which is a point raised by all three groups; this explanation was also offered by Bohnert and Shen [26]. Compatible solutes, including mannitol, may also function as scavengers of oxygen radicals. This idea might be supported by the results of Alia *et al.* [27], where the protection of photosystem II (PSII) in plants expressing *codA* was observed. A second 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 [28], which might also improve the tolerance to salinity stress [14••]. The second commonality is that the levels of glycine betaine production in the transgenic plants are limited by choline, despite little change compared with untransformed plants in the free choline pool. 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 (see discussion below). However, a dramatic increase in glycine betaine 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 [25•]. This limitation was not explored in the transgenic tobacco expressing *E. coli* enzymes CDH and BADH in the cytoplasm [28]. Although these transgenics demonstrated an improved tolerance to salinity, glycine betaine levels were on the order of 1 μmol/g fresh weight as above. Sakamoto and Murata [22] do assert 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) [23]. However, Sakamoto and Murata [22] downplay the limitation of the metabolic pool

of choline on the levels of glycine betaine obtained in the engineered plants. They suggest that the choline-oxidizing activity may be the limiting factor, which seems to be supported by Huang *et al.* [25•] who demonstrated that the levels of glycine betaine correlate with the levels of COX activity measured in each plant. The increase in glycine betaine with exogenous choline (mentioned above) argues against this notion. Stronger evidence for the limitations of choline metabolism has been presented [29••]. By overexpressing 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 glycine betaine levels (+60%) in plants that were already expressing spinach CMO and BADH in the chloroplast. The addition of ethanolamine to the plant growth medium further increased choline and glycine betaine levels, this suggests that the metabolic flux through this pathway is also limited by the supply of ethanolamine. The fact that glycine betaine levels were not increased further may result from the limitation of choline supply to the chloroplast [14••]. PEAMT is itself inhibited by phosphocholine, so further engineering efforts will include the modification of PEAMT to remove this inhibition [29••], increasing the supply of ethanolamine by overexpression of serine decarboxylase, and resolving the compartmentation problem of choline supply and choline oxidation. This latter concern might be overcome either by use of choline oxidation in the cytoplasm or by finding the appropriate transporters to improve choline supply to the chloroplast [14••].

Antioxidant protection

A secondary 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 mitochondrial metabolism during stress. These species cause oxidative damage to different cellular components including membrane lipids, proteins and nucleic acids [30]. 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 tobacco plants overexpressing both GST and GPX displayed improved seed germination and seedling growth under stress [31]. Subsequent studies [32•] demonstrated that in addition to increased GST/GPX activities, the transgenic seedlings contained higher levels of glutathione and ascorbate than wild-type seedlings, displayed 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.

Ion homeostasis

The alteration of ion ratios in the plant results from the influx of sodium through pathways that function in the acquisition of potassium. The stealth of sodium entry results from the similarity between the hydrated ionic radii of sodium and potassium, which makes it difficult for transport proteins to discriminate between the two ions [33]. This discrimination problem also forms the basis for Na^+ toxicity, where key biochemical processes in the plant cell are inhibited by the competition by sodium for potassium-binding sites. The sensitivity to salt of cytosolic enzymes is similar in both glycophytes (salt-sensitive plants) and halophytes (salt-tolerant plants), indicating that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in high salt [34]. Plants could use several strategies to maintain a high K^+/Na^+ ratio in the cytosol: diminishing the entry of Na^+ ions into the cells, extrusion of Na^+ ions out of the cell, and vacuolar compartmentation of Na^+ ions.

Sodium ions can enter the cell through several low-affinity and high-affinity K^+ carriers [35]; among these is AtHKT1 from *Arabidopsis*, which was shown to function as a selective Na^+ transporter and, to a lesser extent, to mediate K^+ transport [36•]. Recently, AtHKT1 was identified as a regulator of Na^+ influx in plant roots. This conclusion was based on the capacity of *hkt1* mutants to suppress Na^+ accumulation and sodium hypersensitivity in an *sos3* (salt-overly sensitive) mutant background [37•], suggesting that AtHKT1 is a salt tolerance determinant that controls the entry of Na^+ into the roots.

Sodium extrusion from plant cells is powered by the operation of the plasma membrane H^+-ATPase. The H^+-ATPase pump generates an electrochemical H^+ gradient that allows plasma membrane Na^+/H^+ antiporters to couple the passive movement of H^+ into the cells, along its electrochemical potential, to the active extrusion of Na^+ [33]. Recently, *AtSOS1* from *A. thaliana* has been shown to encode a plasma membrane Na^+/H^+ antiport that has significant sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria and fungi [38]. Analysis of *AtSOS1*-promoter–GUS transgenic *Arabidopsis* plants showed expression in epidermal cells of the root tip and in parenchyma cells at the xylem/symplast boundary of roots, stems and leaves [39••]. Transgenic *Arabidopsis* plants overexpressing *AtSOS1* displayed enhanced salt tolerance (H Shi, JK Zhu, personal communication) indicating the importance of Na^+ extrusion during salt stress.

The compartmentation of Na^+ into vacuoles provides an efficient mechanism to avert the toxic effects of Na^+ in the cytosol. The transport of Na^+ into the vacuoles is mediated by an Na^+/H^+ antiporter that is driven by the electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes, H^+-ATPase and H^+-pyrophosphatase (H^+-PP_iase) [40]. The overexpression of *AtNHX1*, a vacuolar Na^+/H^+ antiporter from *Arabidopsis*, in *Arabidopsis* resulted in transgenic plants that were able to grow in high salt concentrations [41]. Additional evidence supporting the role of vacuolar transport in salt tolerance has been provided by

A. thaliana plants overexpressing a vacuolar H^+-PP_iase [42••]. Transgenic plants overexpressing *AVP1*, coding for the vacuolar H^+-PP_iase, 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. The paramount role of sodium compartmentation in plant salt tolerance has been further demonstrated in transgenic tomato plants overexpressing AtNHX1, the *A. thaliana* vacuolar Na^+/H^+ antiport [43••]. The transgenic tomato plants grown in the presence of 200 mM NaCl were able to grow, flower, and set fruit. Although the leaves accumulated high sodium concentrations, the tomato fruits displayed very low amounts of sodium [43••]. Similar results were obtained with transgenic *Brassica napus* (Canola) overexpressing AtNHX1 [44••]. Leaves of transgenic plants grown in the presence of 200 mM NaCl accumulated sodium to up to 6% of their dry weight, but the seed yields and oil quality were not affected, demonstrating the potential use of this technology for agricultural use in saline soils.

Conclusions

Twenty years ago, Epstein [45] argued for the development of salt-tolerant crops with truly halophytic responses to salinity (i.e. accumulation of salt) in which the consumable part is botanically a fruit, such as grain or berries or pomes. In these plants, Na^+ would accumulate mainly in their leaves and, because the water transport to the fruits and seeds is mainly symplastic, much of the salt would be screened from these organs. Clearly, the combination of the traits reviewed here is the next step towards the improvement of plant salt tolerance and the generation of salt-tolerant crops. Thus, engineering the accumulation of salt in vacuolated cells, together with the active extrusion of Na^+ from non-vacuolated cells (i.e. young and meristematic tissue), will allow the maintenance of a high cytosolic K^+/Na^+ ratio. In combination with the enhanced production of compatible solutes, this will generate transgenic crop plants able to tolerate and grow in high soil salt concentrations.

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The authors overexpressed the plant vacuolar H^+-PP_iase in transgenic *Arabidopsis* plants. The transgenic plants displayed enhanced tolerance to drought and salt-stress and accumulated more Na^+ and K^+ in their leaves than wild-type plants. Measurements of isolated tonoplast vesicles showed that vesicles from transgenic plants had enhanced cation uptake, suggesting that the increased H^+-PP_iase activity in the transgenic plants provided additional driving force for the operation of vacuolar H^+/cation antiports.

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The authors showed that transgenic tomato plants overexpressing *AtNHX1,* a vacuolar Na^+/H^+ antiporter from *A. thaliana*, were able to grow, flower and produce fruit in the presence of 200 mM NaCl. Although the leaves of the transgenic plants accumulated high sodium concentrations, the tomato fruits displayed very low sodium content.

44. •• Zhang HX, Hodson JN, Williams JP, Blumwald E: **Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation.** *Proc Natl Acad Sci USA* 2001, **98**:12832-12836.

Transgenic *Brassica* plants overexpressing a vacuolar Na^+/H^+ antiporter were able to grow in the presence of 200 mM NaCl and produced seed yields similar to those produced by wild-type plants grown under normal conditions. Plant lipid and seed oil analyses revealed that the transgenic plants displayed little signs of stress or acclimation to high NaCl concentrations. Analysis of oil quality showed no significant differences between seeds from wild type and transgenic plants grown at low or high salinity.

45. Epstein E: **Crops tolerant to salinity and other mineral stresses.** In *Better Crops for Food, Ciba Foundation Symposium.* Edited by Nugent J, O'Connor M. London: Pitman; 1983:61-82.