Developing salt-tolerant crop plants: challenges and opportunities

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Soil salinity, one of the major abiotic stresses reducing agricultural productivity, affects large terrestrial areas of the world; the need to produce salt-tolerant crops is evident. Two main approaches are being used to improve salt tolerance: (i) the exploitation of natural genetic variations, either through direct selection in stressful environments or through mapping quantitative trait loci and subsequent marker-assisted selection; and (ii) the generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to affect the degree of salt stress tolerance. Here, we discuss the challenges and opportunities provided by recently developed functional tools for the development of salt-tolerant crops.

Introduction

Agricultural productivity is severely affected by soil salinity because salt levels that are harmful to plant growth affect large terrestrial areas of the world. The damaging effects of salt accumulation in agricultural soils have influenced ancient and modern civilizations. It is estimated that 20% of the irrigated land in the world is presently affected by salinity [1]. 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 over the next 20 years, and the challenge of maintaining the world food supplies. Although famine in the world nowadays is caused by complex problems and not just by insufficient food production, the gains in agricultural output provided by the Green Revolution have reached their ceiling whereas the world population continues to rise. Therefore, increasing the yield of crop plants in normal soils and in less productive lands, including salinized lands, is an absolute requirement for feeding the world.

The degradation of the agricultural land and water supplies is the result of intensive agricultural practices employed in developed and developing countries. Ideally, these practices should be changed to a more rational use of land and water resources; however, this change will not occur in the foreseeable future. For example, mixed cropping with perennials and trees would alleviate the accumulation of sodium and other salts in the upper soil layers. Nonetheless, this kind of change in farming systems and the development of new products is likely to be a long and

difficult process because it will require the use of new land and will not address the problem of growing crops in land that is already compromised. The development and use of crops that can tolerate the high levels of salinity in the soils would be a practical contribution towards addressing the problem.

Efforts to improve crop performance under environmental stresses have not been that fruitful because the fundamental mechanisms of stress tolerance in plants remain to be completely understood. Twenty-five years ago Emanuel Epstein [2] described the technical and biological constraints to solving the problem of salinity. Although there has been some success with technical solutions to the problem, the biological solutions have been more difficult to develop because a pre-requisite for the development of salt-tolerant crops is the identification of key genetic determinants of stress tolerance. The existence of salt-tolerant plants (halophytes) and differences in salt tolerance between genotypes within salt-sensitive plant species (glycophytes) indicates that there is a genetic basis to salt response.

Two basic genetic approaches that are currently being used to improve stress tolerance include: (i) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of quantitative trait loci (QTLs – regions of a genome that are associated with the variation of a quantitative trait of interest) [3–5] and subsequent marker-assisted selection, and (ii) generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to affect the degree of salt stress tolerance. We will discuss these approaches in some detail, focusing on the recent experimentation with transgenic plants that has led to increased salinity tolerance, with emphasis on the areas of ion homeostasis. The role of antioxidants, osmoregulation, signaling and transcriptional control have been recently reviewed [6–9] and, thus, will not be dealt with here.

Marker-assisted breeding

The direct selection of superior salt-tolerant genotypes under field conditions is hindered by the significant influence that environmental factors have on the response of plants to salinity [10]. There is also evidence supporting the notion that salt tolerance is a complex trait involving the function of many genes [3,4]. Salt tolerance in plants appears to be a developmentally regulated process and the tolerance of the plants at one stage of development is not always correlated with tolerance at other

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stages [3,4,11,12]. For example, in tomato, barley, corn, rice and wheat, salt tolerance tends to increase with the age of the plant [3]. QTLs associated with salt tolerance at the germination stage in barley [13], tomato [14] and *Arabidopsis* [15] were different from those QTLs associated with salt tolerance at the early stage of growth; the plants selected by their ability to germinate at high salinity did not display similar salt tolerance during vegetative growth.

The development of molecular biology techniques has enabled the development of DNA markers that can be used to identify QTLs. The use of QTLs has improved the efficiency of selection, in particular for those traits that are controlled by several genes and are highly influenced by environmental factors [4]. QTLs and marker-assisted selection provide several advantages over direct phenotypic screening, particularly because the PCR-based methodologies used to detect the markers reduce the time needed to screen individuals and reduce the impact of environmental effects on the trait under study. There is considerable evidence to support the view that salt tolerance and its sub-traits are determined by multiple QTLs and that both additive and dominance effects are important in the inheritance of many of the traits associated with salt tolerance [3,4,16]. The development of high-density DNA maps that incorporate microsatellite markers, RFLP (restriction fragment-length polymorphisms) and AFLP (amplified fragment-length polymorphisms), and advances in marker-assisted selection techniques will facilitate pyramiding traits of interest to attain substantial improvement in crop salt tolerance.

Salt tolerance using transgenic approaches

Physiologically, salinity (i) imposes an initial water-deficit that results from the relatively high solute concentrations in the soil, (ii) causes ion-specific stresses resulting from altered K^+/Na^+ ratios and (iii) leads to build up in Na^+ and Cl^- concentrations that are detrimental to plants. Plants respond to salinity using two different types of responses. Salt-sensitive plants restrict the uptake of salt and adjust their osmotic pressure by the synthesis of compatible solutes (e.g. proline, glycinebetaine and sugars) [12]. Salt-tolerant plants sequester and accumulate salt into the cell vacuoles, controlling the salt concentrations in the cytosol and maintaining a high cytosolic K^+/Na^+ ratio in their cells [17]. Ion exclusion mechanisms could provide a degree of tolerance to relatively low concentrations of NaCl but would not work at high concentrations of salt, resulting in the inhibition of key metabolic processes and concomitant growth inhibition. Here, we discuss processes that contribute to the establishment of cellular ion homeostasis.

Although Na^+ is required in some plants, particularly halophytes, a high concentration of NaCl is toxic and affects plant growth [17]. The alteration of ion ratios in plants is due to the influx of Na^+ through pathways that function in the acquisition of K^+ [18]. The sensitivity of cytosolic enzymes to salt 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 soils with a high concentration of salt [17]. Strategies that plants could use 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) [19], a rise in extracellular Na^+ concentration would establish a large electrochemical gradient favoring the passive transport of Na^+ into the cells.

Three classes of low-affinity K^+ channels have been identified. Inward rectifying channels (KIRC), such as AKT1 [20], activate K^+ influx upon plasma-membrane hyperpolarization and they display a high K^+/Na^+ selectivity ratio (Figure 1). A knockout mutant of *AKT1* in *Arabidopsis* (*akt1-1*) displayed similar sensitivity to salt to that of the wild type, suggesting that this channel does not play a role in Na^+ uptake [21]. K^+ outward rectifying channels (KORCs) could play a role in mediating the influx of Na^+ into plant cells. KORC channels show a high selectivity for K^+ over Na^+ in barley roots [22], and a somewhat lower K^+/Na^+ selectivity ratio in *Arabidopsis* root cells [23]. These channels, which open during the depolarization of the plasma membrane (i.e. upon a shift in the electrical potential difference to more positive values), could mediate the efflux of K^+ and the influx of Na^+ ions [23]. Voltage-independent, non-selective cation channels (NSCC) in plant plasma membranes have been reported [24,25]. These channels have a relatively high Na^+/K^+ selectivity, are not gated by voltage and provide a pathway for the entry of Na^+ into plant cells [26].

Sodium ions can enter the cell through several low- and high-affinity K^+ carriers. Among these is AtHKT1 from *Arabidopsis*, which has been shown to function as a selective Na^+ transporter and, to a lesser extent, to mediate K^+ transport [27] (Figure 1). AtHKT1 was identified as a putative 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 a *sos3* (salt overly sensitive) mutant background [28]. Other studies have shown that loss-of-function mutations in the AtHKT1 gene lead to over accumulation of Na^+ in the shoots, increasing the sensitivity of the plant to Na^+ [29,30]. Based on these results, the authors proposed that AtHKT1 played a role in long-distance Na^+ transport and Na^+ circulation in the plant, with AtHKT1 mediating Na^+ loading into the leaf phloem and Na^+ unloading from the root phloem sap [30].

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^+ [31] (Figure 1). *AtSOS1* from *Arabidopsis thaliana* has been shown to encode a plasma membrane Na^+/H^+ antiporter with significant sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria and fungi [32]. 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 [33] (Table 1). Similar results were obtained when the
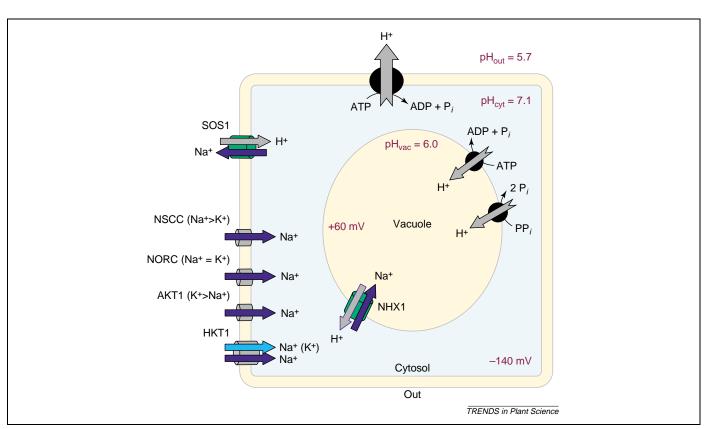
Figure 1. Schematic representation of Na^+ transport in plant cells. Electrogenic H^+ transport (H^+-ATPase in the plasma membrane and vacuolar membrane, H^+-PPiase in the vacuolar membrane) generates gradients of pH and electrical potential difference across the cell and vacuolar membranes. Na^+ ions enter the cell via different channels (AKT1, NORC, NSCC) or carriers (HKT1) 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.

plasma membrane Na^+/H^+ antiporters SOD2 from *Schizosaccharomyces pombe* and nhaA from *Escherichia coli* were overexpressed in *Arabidopsis* [34] and rice [35], respectively. 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

Table 1. Salt tolerance in transgenic plants expressing genes involved in ion transporters

Gene	Gene product	Source	Cellular role(s)	Target plant	Parameter studied	Refs
AtNHX1	Vacuolar Na^+/H^+ antiporter	*Arabidopsis*	Na^+ vacuolar sequestration	*Arabidopsis*	Biomass	[36]
AtNHX1	Vacuolar Na^+/H^+ antiporter	*Arabidopsis*	Na^+ vacuolar sequestration	Tomato	Biomass, fruit yield	[37]
AtNHX1	Vacuolar Na^+/H^+ antiporter	*Arabidopsis*	Na^+ vacuolar sequestration	*Brassica napus*	Biomass, oil production	[38]
AtNHX1	Vacuolar Na^+/H^+ antiporter	*Arabidopsis*	Na^+ vacuolar sequestration	Maize	Biomass	[42]
AtNHX1	Vacuolar Na^+/H^+ antiporter	*Arabidopsis*	Na^+ vacuolar sequestration	Wheat	Biomass, grain yield	[43]
GhNHX1	Vacuolar Na^+/H^+ antiporter	*Gossypium hirsutum*	Na^+ vacuolar sequestration	Tobacco	Biomass	[41]
AgNHX1	Vacuolar Na^+/H^+ antiporter	*Atriplex gmelini*	Na^+ vacuolar sequestration	Rice	Biomass	[39]
OsNHX1	Vacuolar Na^+/H^+ antiporter	*Oryza sativa*	Na^+ vacuolar sequestration	Rice	Growth, ion content	[40]
BnNHX1	Vacuolar Na^+/H^+ antiporter	*Brassica napus*	Na^+ vacuolar sequestration	Tobacco	Growth, seed yield	[44]
HbNHX1	Vacuolar Na^+/H^+ antiporter	*Hordeum brevisubculatum*	Na^+ vacuolar sequestration	Tobacco	Biomass	[45]
AtSOS1	Plasma membrane Na^+/H^+ antiporter	*Arabidopsis*	Na^+ extrusion	*Arabidopsis*	Biomass	[33]
SOD2	Plasma membrane Na^+/H^+ antiporter	*Schizosaccharomyces pombe*	Na^+ extrusion	*Arabidopsis*	Biomass, photosynthesis	[34]
nhaA	Plasma membrane Na^+/H^+ antiporter	*Escherichia coli*	Na^+ extrusion	Rice	Biomass, ion content	[35]
AVP1	Vacuolar H^+-pyro phosphatase	*Arabidopsis*	Vacuolar acidification	*Arabidopsis*	Biomass	[46]

of protons generated by the vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-PPiase [18]. The overexpression of *AtNHX1,* a vacuolar Na^+/H^+ antiporter, in *Arabidopsis* resulted in transgenic plants that were able to grow in high concentrations of salt [36]. The paramount role of Na^+ compartmentation in plant salt tolerance has been further demonstrated in transgenic tomato plants overexpressing AtNHX1 [37]. 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 concentrations of sodium, the tomato fruits displayed low amounts of sodium [37]. Similar results were obtained with transgenic *Brassica napus* (canola) overexpressing *AtNHX1* [38]. Sodium accumulated in the leaves of transgenic plants grown in the presence of 200 mM NaCl formed up to 6% of the dry leaf weight, but the seed yields and oil quality were not affected, demonstrating the potential use of this technology for agricultural use in saline soils. Similar results have been reported in other species. The introduction of a vacuolar Na^+/H^+ antiporter from the halophyte *Atriplex gmelini* conferred salt tolerance in rice [39]. The overexpression of the rice vacuolar Na^+/H^+ antiporter (*OsNHX1*) in rice also conferred salt tolerance to the transgenic plants [40]. Recently, several reports have further demonstrated the importance of vacuolar Na^+ compartmentation in plant salt tolerance [41–45]. The overexpression of AtNHX1 resulted in enhanced salt tolerance in transgenic maize [42] and wheat [43]. The overexpression of *BnNHX1* (*Brassica napus*), *HbNHX1* (barley) and *GhNHX1* (cotton) resulted in enhanced salt tolerance in transgenic tobacco [41,44,45]. Additional evidence supporting the role of vacuolar transport in salt tolerance has been provided by *Arabidopsis* plants overexpressing a vacuolar H^+-PPiase [46]. Transgenic plants overexpressing *AVP1,* coding for the vacuolar H^+-pyrophosphatase, showed 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 an additional driving force for vacuolar sodium accumulation via the vacuolar Na^+/H^+ antiporter.

Challenges

The assessment of salt tolerance in transgenic experiments as described above has been mostly carried out using a limited number of seedlings or mature plants in laboratory experiments. In most of the cases, the experiments were carried out in greenhouse conditions where the plants were not exposed to those conditions that prevail in high-salinity soils (e.g. alkaline soil pH, high diurnal temperatures, low humidity, presence of other sodic salts and elevated concentrations of selenium and/or boron). The salt tolerance of the plants in the field needs to be evaluated and, more importantly, salt tolerance needs to be evaluated as a function of yield. The evaluation of field performance under salt stress is difficult because of the variability of salt levels in field-conditions [47,48] and the potential for interactions with other environmental factors, including soil fertility, temperature, light intensity and water loss due to transpiration. Evaluating tolerance is also made more complex because of variation in sensitivity to salt during the life cycle. For example, in rice, grain yield is much more affected by salinity than is vegetative growth [49]. In tomato, the ability of the plants to germinate under conditions of high salinity is not always correlated with the ability of the plant to grow under salt stress because both are controlled by different mechanisms [50], although some genotypes might display similar tolerance at germination and during vegetative growth [51]. Therefore, 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 [52], there is an urgent need to test these successes in other crops. Rice has the advantages of being both the model monocot and an important crop. However, this is not the case when transgenes are tested with tobacco or *Arabidopsis* [4,52]. There are several 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 because many of the stress tolerance assays used by basic researchers involve using nutrient-rich media (which in some cases include sucrose). This type of screen is unlikely to have a relationship to field performance. Third, because saline soils are often complex and can include NaCl, $CaCl_2$, $CaSO_4$, Na_2SO_4, high boron concentrations and alkaline pH, plants that show particular promise will eventually have to be tested in all these environments.

Conventional breeding programs for selecting salt-tolerant genotypes have met with limited success. This lack of success is in part because breeders prefer to evaluate their genetic material in ideal conditions. This issue is becoming more urgent because of the growing interest of commercial seed companies in making salt-tolerant crops. From a business perspective, for plant breeding companies to invest in the development of new varieties with enhanced stress tolerance, there will always be the question of whether investing in the development of these cultivars is worth the effort. There is no benefit in developing salinity-tolerant plants unless there are economic drivers that will allow the plant to be competitively productive with non-saline-tolerant plants growing on uncompromised soil. The viewpoint of basic researchers might differ from this because, for the researchers, the actual, albeit small, increase in salt tolerance is worth the effort.

In evaluating the possibility of improving stress tolerance in plants, there are several elements that should be considered. First, although 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 [52]. 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 [53,54].

Second, although there have been several successes in producing abiotic stress-tolerant tobacco and *Arabidopsis* plants, we now need to begin introducing 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. Plant breeders have already developed many genotypes that have been selected for traits such as high yields, enhanced resistance to pathogens and improved tolerance to abiotic stress. The use of these already selected germplasms for transformation with the different genes identified should be emphasized. It is likely that the effectiveness of a specific transgene will be based on the specific genetic background into which it is transformed. One component of this is the well known phenomena of 'position effect', but the ability of a transgene to work might well be determined by the overall genetic background, independent of the chromosomal location of the transgene, referred to as the 'transgene combining ability'.

Opportunities

Although progress in improving stress tolerance has been relatively slow, there are several opportunities and reasons for optimism. Over the past ten years, several functional tools have been developed that have enabled us to dissect many of the fundamental questions associated with stress tolerance. These include: (i) the development of molecular markers for gene mapping and the construction of associated maps, (ii) the development of EST libraries, (iii) the complete sequencing of plant genomes, including *Arabidopsis*, rice and maize, (iv) the production of T-DNA or transposon-tagged mutagenic populations and (v) the development of several forward genetics tools that can be used in gene function analysis such as TILLING [55]. 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. In addition, we should be aware that the overexpression (or the suppression) of a particular gene not only affects the function of the gene product but also affects different pathways. For example, transcriptional profile analyses of AtNHX1 knockout plants growing in the presence or absence of salt revealed that, in addition to the changes in the expression of the vacuolar Na^+/H^+ antiporter, the expression of genes encoding proteins associated with intravesicular trafficking and trafficking to the nucleus and the Golgi apparatus were also affected. This supports the notion that, in addition to its role in the accumulation of Na^+ into the vacuole, AtNHX1 plays a significant role in protein trafficking and protein targeting, probably via the regulation of the acidic intravesicular pH [56].

Research on the physiology of salt tolerance has demonstrated that the overall trait is determined by several sub-traits, any of which can in turn be determined by several genes. A combination of genome-wide patterns of expression (DNA arrays) and QTL mapping could provide important information with regards to how the expression of genes associated with the QTL region are affected by a particular environmental or experimental condition. Once particular regions or genes are identified, their overexpression in the salt-sensitive lines and/or their silencing in the salt-tolerant lines could provide a further assessment of the physiological role of the gene products and, more importantly, the identification of other regions of the genome interacting with the QTL under study. We believe that by comparing different genes and genetic combinations, researchers will be able to advance the field more quickly and develop salt-tolerant germplasms.

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