Options for Developing Salt-tolerant Crops

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Abstract. Soil salinization is an increasing problem worldwide and is often intensified by irrigation. Unfortunately, few new crop cultivars have been developed resistant to saline soils, a consequence, in part, of the complexity of plant responses to salt stress. There are now, however, several non-traditional options to improving salt tolerance as a result of recent progress in better understanding the mechanisms involved. These mechanisms include 1) exclusion of Na⁺ and Cl⁻ from plant tissues; 2) inclusion of these ions in inert compartments or tissues; and/or 3) some means of osmotic adjustment with solutes that are compatible with the metabolic machinery of the cell. Although there are very few horticultural examples, several lines of evidence indicate that reductions in salt sensitivity through exclusion or inclusion can be achieved by single gene modifications of the ion transport system. Similarly, single genes resulting in osmotic adjustment with solutes compatible with the metabolic machinery of the cell have resulted in significant increases in salt tolerance. Recent advances in sequencing, use of quantitative trait loci, and marker-assisted selection promise to provide other options for improving salt tolerance.

BACKGROUND AND IMPORTANCE OF THE SALINITY PROBLEM

Although estimates vary widely (Rozema and Flowers, 2008), soil salinization is a major factor reducing crop yields in the United States and globally. More than 800 million ha of land is salt-affected, which is over 6% of the world's land area (FAO Land and Plant Nutrition Management Service; Flowers and Yeo, 1995; Munns, 2009; Rengasamy, 2010). There are several causes. In dryland agriculture without irrigation in areas that receive rainfall less than 200 to 300 mm/year, capillarity allows salts in groundwater to reach the surface. This is particularly a problem in less-developed countries with large and growing populations in arid climatic zones. Dryland (or non-irrigated) salinization is also common as a result of rising water tables over essentially saline sub-soils as a result of clearance of trees or other deep-rooted species, usually to make land available for rain-fed agriculture. This process has been well documented with land clearance in Australia (Rengasamy, 2006). Rising water tables also lead to water-logging with oxygen deficiencies that reduce the ability of roots to exclude salts (Barrett-Lennard, 2003).

Salinity problems frequently result from irrigation. Indeed, it is an ancient problem that confronted the Sumerians (Epstein and Bloom, 2005). Estimates vary, with some indicating that up to 50% of all irrigated lands may be salt-affected (Flowers, 1999; Szabolcs, 1989), and these numbers are expected to increase. Irrigation-induced (secondary) salinity effects are particularly problematic in arid

climatic zones. River systems in these regions with extensive irrigated agriculture supply the water for irrigation but also the salts dissolved in it. At 1 ppm of salt, an acre foot of irrigation water contains over 1 ton of salt (1 meter ha contains over 8 MT), and Colorado river water in Southern California may be as high as 900 ppm (Anon, 1990). Under such conditions, salinization may be irreversible because fresh water limitations preclude leaching accumulated salts, particularly as urbanization and industrialization heighten competition for high-quality water (Evans, 1998; Rains and Goyal, 2003). Leaching is questionable because the leachate/saltwater created can cause further damage. Irrigation by drawing down rivers and aquifers in coastal areas may also lead to seawater incursions and tidal intrusions (Flowers, 1999).

Nonetheless, irrigation has been a major contributor to increases in global food production and to over 30% of total agricultural production (Munns and Tester, 2008). Thus, secondary salinization of irrigated lands is of major concern for global food production. In horticulture, although the scale of production is often smaller than that for the major agricultural crops, higher crop values justify irrigation investments, and intense cultivation in protected environments almost always requires irrigation with consequent salinity problems (Flowers, 1999). Elevated salt levels are also a problem with use of alternative water sources and reclaimed and non-potable waters for irrigation in production and maintenance of landscape plants in arid environments (Niu and Cabrera, 2010). In short, wherever water is scarce and droughts are recurring, the many causes of soil salinity are major constraints to crop productivity (Bressanet al., 2008; Flowers and Yeo, 1995; Kronzucker and Britto, 2011; Läuchli and Lüttge, 2002; Munns, 1993).

Given the dimensions of the problem and the importance of sustainable irrigation to crop production, it is likely that salinization will become an even more significant issue in world agriculture during the 21st century. Not surprisingly, a multitude of publications have addressed the many dimensions of the

problem, including numerous reviews in the past few years (Apse and Blumwald, 2007; Ashraf et al., 2008; Bressan et al., 2008; Flowers and Colmer, 2008; Kronzucker and Britto, 2011; Mittler and Blumwald, 2010; Munns, 2009; Munns and Tester, 2008; Niu and Cabrera, 2010; Riadh et al., 2010; Tester and Langridge, 2010).

IF SUCH A PROBLEM, WHY SO FEW NEW CROPS AND CULTIVARS RESISTANT TO SALINE SOILS?

Why so little success? In the recent past, salinity was not seen as a major problem because salinized fields could be abandoned and replaced by others. Thus, there was little regional or global impact, even if there were major local effects. As a local problem, the conventional approach, e.g., drainage engineering, was the usual "solution" to facilitate leaching the salt below the root zone with high-quality water; however, this is often unfeasible in developing countries and where irrigation water is limited. Alternative approaches include more sophisticated irrigation techniques to minimize build-up of salt in the root zone, e.g., trickle and drip systems and use of amendments such as calcium sulfate to displace sodium adsorbed on the soil-cation exchange complex. However, these approaches often confront environmental, social, economic, and even legal challenges (Epstein and Bloom, 2005; National Research Council, 1989). The lack of success of breeding programs in developing commercially successful salt-tolerant crops is also often the result of breeders' preference for evaluating their genetic material under idealized conditions (Rengasamy, 2006).

COMPLICATIONS: TWO COMPONENTS OF SALT STRESS

There are two major components of salt stress (Munns, 2002). The first is osmotic, i.e., obtaining water from a soil of highly negative osmotic potential. Thus, there is an inseparable relationship between water and salt stress in which the first response to salt stress is

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dehydration/drought. This has implications for breeders, physiologists, and molecular biologists. What should they select for and at what stage of development: at stand establishment, at flowering, at fruit or seed set, at seed fill, or fruit growth? Short-term selection procedures would likely reflect a response to the osmotic strength of the external solution instead of a response to the toxic effect of the salt.

The second component is the result of specific ion effects. Although the focus is usually on dealing with high concentrations of potentially toxic sodium ions, carbonate and chloride ions are also problems (and all of these can result in other nutritional imbalances or deficiencies). Furthermore, these effects are often reduced when Ca^{++} levels are high. Interactions between calcium levels and salt stress are common. Thus, a related complication is "sodicity," the presence of Na^+ relative to Ca^{++} and Mg^{++} in the soil: expressed as the sodium adsorption ratio because most cations in the soil are attracted to the negative charges of clays. Salinity (sodicity) effects may, however, also involve different ion species, i.e., Na^+ , Cl^- , HCO_3^- , PO_4^{3-} , Ca^{2+} , Mg^{2+} , SO_4^{2-} , and/or borate, and their interactions.

The situation is further complicated by the complexity of organismal responses to water deficit as Bohnert et al. (1995) and Bray (1993, 1997) have indicated. Responses begin with stress perception followed by one or more signal transduction pathways and the responses include changes at the cellular, physiological, and developmental levels. The responses also depend on severity and duration of the stress, genotype, developmental stage, and environmental factors. Furthermore, at the cellular level, water deficit may result from several stresses: drought, salt, and low or high temperature. This is confounded by terrestrial plants normally experiencing and responding to water stress almost daily. Separating normal and transient responses from abnormal longer-term drought effects is a challenge. As just one example of the complexity, in *Arabidopsis* in response to salt stress, numerous transcripts were up- or downregulated (1793 and 1446, respectively, with a fold difference greater than two, $P < 0.05$) (Chan et al., 2011).

All this makes it difficult to uncover those responses that specifically enhance salt stress tolerance. However, the salt tolerance character is common. Marine algae inhabit an environment that is ≈ 0.5 M NaCl, and coastal estuaries and salt marshes are often unusually productive (Bertness et al., 2004). An analysis (Flowers et al., 2010) of the evolution of salt tolerance in higher vascular plants indicated that salt tolerance (ability to tolerate at least 200 mM NaCl) has independently and repeatedly evolved many different times in angiosperms and in different ways. Nonetheless, there are some commonalities. Tolerance (to high salt levels in plant tissues) almost always requires the combination of several different traits: accumulation and compartmentation of ions for osmotic adjustment; the synthesis of compatible solutes; the ability to accumulate essential nutrients (especially

K^+) in the presence of high concentrations of the ions generating salinity (particularly Na^+); the ability to limit uptake and the entry of these saline ions into the transpiration stream and into the cytosol; and the ability to continue to regulate transpiration in the presence of high concentrations of Na^+ and Cl^- (Flowers and Colmer, 2008). There is also the need to keep Ca^{++} levels low in the cytosol and relatively high in the apoplast (Epstein and Bloom, 2005). Just as there is division of ions between the cytosol and the vacuole and apoplast, there may also be division of the whole plant into prioritized and non-prioritized tissues, e.g., between younger and older leaves, or mesophyll and epidermis, or fruits and meristems versus older leaves (Amtmann and Leigh, 2010).

ANOTHER COMPLICATION—FEW CROP PLANTS DEMONSTRATE TRUE SALT TOLERANCE

Although a few horticultural crops are moderate or true halophytes, e.g., tomato (Solanaceae), celery (Apiaceae), olive (Oleaceae), date palm (Arecaceae), asparagus (Liliaceae), and beet (Chenopodiaceae), most are sensitive, and some are very sensitive (glycophytes, non-halophytes), e.g., pea, bean, chickpea, onion, citrus, and peach and related stone fruits. Unfortunately, although there are numerous comparisons of salt injury among ornamentals, only a little information is available on variation within species or their physiology when stressed (Kotuby-Amacher et al., 2000; Niu and Cabrera, 2010; Robins et al., 2009; Zhou et al., 2010). The term tolerance here may not be appropriate because glycophytes may use exclusion to some extent and avoidance mechanisms such as reabsorption of Na^+ from the xylem stream and its recirculation down to the roots. For example, the ability of citrus to grow in somewhat saline soils is generally associated with exclusion or limited transport to the leaves by specific rootstocks rather than tolerance of ions in leaf tissues (Storey and Walker, 1998).

For a number of horticultural crops, natural variation exists within the species and their close relatives, e.g., strawberry (Hancock and Bringham, 1979), but with few exceptions, e.g., tomato (Foolad, 2004) and agronomic crops like rice, wheat, and barley (Islam et al., 2007; Nevo and Chen, 2010); this is largely unexplored. For reasons already mentioned, screening is difficult, particularly for yield, in which small, repeatable, and quantifiable differences are important. Introgressing salt tolerance into commercially important genotypes is invariably slow regardless of the species, but perennial woody crops present particular problems. As Munns (2009) has indicated, more targeted and feasible selection techniques are required. Furthermore, given the obvious complex polygenic nature of salt tolerance, knowledge of the target environment and understanding of the genetic basis for improvement will be necessary to develop appropriate screening methods.

Nonetheless, although salt tolerance is quite complex, and perhaps not amenable

to simple approaches to breeding problems, both classical and non-traditional ("biotechnological") approaches are now common. Bressan et al. (2008) have outlined a list of strategies for enhancing salinity stress tolerance in crop species. These include traditional breeding with selection for yield, mutation breeding, screening within phenotype, and introducing germplasm from wild species into the crop species. There has been some progress with these approaches (Nevo and Chen, 2010; Omielan et al., 1991; Schachtman et al., 1989; Witcombe et al., 2008). A less traditional approach would entail generating new halophytic crops from halophytic wild species. Again, there are few, if any, horticultural examples. Nontraditional molecular approaches already underway include transgenic modifications and creation of genetic maps for quantitative trait locus (QTL) analyses and identification of candidate genes, and here there has been significant progress. Indeed, performance of transgenic plants under controlled conditions has sometimes been dramatic. Undergoing rapid development is transcript/protein/metabolite profiling of model and crop species and their stress-tolerant relatives so as to identify candidate genes and to develop better screening methods. Still in its infancy is the systems biology approach, which combines characters from mutation screening, genome sequencing, reverse and forward genetics, and computational tools into QTL-characterized backgrounds.

MAJOR OPTIONS FOR CROP IMPROVEMENT

Despite the complexities of salt tolerance, there are several major options for crop improvement. As already indicated, tolerance usually involves a combination of several mechanisms that appear to be nearly ubiquitous: 1) exclusion of Na^+ and Cl^- ; or 2) inclusion of these ions in inert compartments or tissues; and 3) exclusion/inclusion coupled with some means of osmotic adjustment with solutes that are compatible with the metabolic machinery of the cell. Accordingly, most of the examples of transgenic plants that demonstrate major improvements in tolerance involve transformation with single genes controlling one of these mechanisms. A comprehensive and frequently updated list of these is available at <http://www.plantstress.com/>.

Exclusion and inclusion are part of the ubiquitous and highly conserved ion homeostatic mechanisms present in all plants (glycophytes and halophytes). There is no sharp line between inclusion and exclusion/extrusion and some plants demonstrate a combination of both. With a two-phase growth response (first to drought and then to ion toxicity) (Munns, 2002), inclusions may experience reduced growth as a result of premature senescence of old leaves and thus reduced supply of assimilates. However, the result is to remove salt from the plant (more specifically, from the cytosol). Regardless of degree, salt tolerance of both halophytes and glycophytes is

dependent on the homeostatic mechanisms that control net ion uptake across the plasma membrane and compartmentation into the vacuole. Furthermore, cells of virtually all plants possess the capability to sense and respond to a saline environment (Amtmann and Leigh, 2010). Ion concentrations and transport are tightly controlled and thus the question is to what extent do halophytes have unique capabilities that distinguish them from glycophytes. Salt-sensitive plants generally may tolerate moderate salinity because of root mechanisms that reduce movement of harmful ions to the shoot. Halophytes generally have a greater capacity to tolerate vacuolar compartmentalization of ions in leaf cells (Flowers et al., 2010).

MANIPULATING ION HOMEOSTASIS—TWO APPROACHES

Manipulating ion homeostasis would appear formidably complex. In *Arabidopsis*, for example, transport proteins represent at least 5% of the genome (Maser et al., 2001), and there are 35 K⁺ transporters. Conductance and selectivity of many of these transporters are also known to be regulated by various factors: substrates and levels of other ions (particularly Ca²⁺), membrane electrical potential (some are depolarization- or hyperpolarization-activated and others are voltage-insensitive), and response to ligands and stimuli (gated by cyclic nucleotides or amino acids or activated by reactive oxygen species) (Kronzucker and Britto, 2011). However, several lines of evidence now indicate that tolerance can be achieved by single gene modifications of the ion transport system. After research showing that expression of an *Arabidopsis* vacuolar H⁺-pyrophosphatase could improve salt tolerance when expressed in yeast (Gaxiola et al., 1999), this gene was then overexpressed in *Arabidopsis* (Gaxiola et al., 2001) and more recently in cotton (Pasapula et al., 2011) and bentgrass (Li et al., 2010b) and plants were much more resistant to high concentrations of NaCl and drought than their wild types. Transgenic plants accumulated more Na⁺ and K⁺ in their leaf tissues than the wild type. Moreover, direct measurements on vacuolar membrane vesicles derived from the *AVPI* transgenic plants and from wild type demonstrated that vesicles from transgenic plants had enhanced cation uptake.

Similarly, Blumwald and collaborators (Apse et al., 2003; Zhang and Blumwald, 2001) showed that overexpression in tomato of a specific vacuolar Na⁺/H⁺ antiporter, AtNHX1, could dramatically improve vegetative growth and fruit yield. Comparable results have since been obtained in a number of crops, e.g., *Brassica* (Zhang et al., 2001), rice (Ohta et al., 2002), maize (Yin et al., 2004), wheat (Xue et al., 2004), cotton (He et al., 2005), tobacco (Lu et al., 2005), and sugar beet (Liu et al., 2008). For a perspective on this antiporter's potential in plant breeding, see Hanana et al. (2009). However, other attempts to express similar Na⁺/H⁺ antiporters have not been so successful, thus questioning a broad role for vacuolar NHX1-like proteins

as effective Na⁺ scavengers in planta (Leidi et al., 2010). On the other hand, in *Arabidopsis*, a similar transporter, AtHKT1;1, drives elevated leaf Na⁺. This has been previously linked to elevated salinity tolerance, and geographical distribution indicates its enrichment in populations associated with saline and coastal soils, thus providing genetic evidence supporting a role for AtHKT1;1 in local adaptation to potentially saline-impacted environments (Baxter et al., 2010).

In a related approach, *AtHKT1;1* improved sodium (Na⁺) exclusion and salinity tolerance when expressed in *Arabidopsis* root cortical and epidermal cells (Moller et al., 2009) and in rice when expressed and targeted to the mature root stele (Plett et al., 2010). Constitutive 35S-promoted expression did not have that effect. Interestingly, in the transgenic *Arabidopsis* plants overexpressing *AtHKT1;1* in the cortex and epidermis, the native *AtHKT1;1* gene responsible for Na⁺ retrieval from the transpiration stream was also upregulated as was expression of the vacuolar pyrophosphatases (in both *Arabidopsis* and rice) necessary to move the additional stored Na⁺ into the vacuoles of these cells.

In another approach, Zhu and colleagues (Qiu et al., 2002; Wu et al., 1996; Zhu, 2002) focused on the SOS signal transduction pathway in which the key ion transport step is SOS-1, a plasma membrane Na⁺/H⁺ antiporter that appears to control long-distance sodium transport in plants (Shi et al., 2002). Although SOS-1 is only a part of a complex signaling pathway that controls sodium uptake by the root system (Bressan et al., 2008; Yang et al., 2009), overexpression of *SOS-1* improved salt tolerance in *Arabidopsis* (Shi et al., 2003). It is also interesting that the *AtSOS1* transcript is upregulated under salt stress, and the stability of the transcript itself is maintained in the presence of NaCl (Shi et al., 2000; Ward et al., 2003). The SOS-1 protein, however, appears to play other roles in addition to Na⁺ transport (Kronzucker and Britto, 2011; Oh et al., 2010), which may confound interpretation of the ectopic expression results.

A THIRD APPROACH: OSMOTIC ADJUSTMENT WITH COMPATIBLE SOLUTES

Osmotic adjustment or osmoregulation can be achieved by several means, e.g., through succulence (of leaves), salt and solute accumulation, or shedding of older leaves, or a combination of these factors. More frequently,

however, adjustment involves compatible solutes, classes of compounds that can accumulate in the cytosol without damaging enzymes. See Loescher and Everard (2000) and Rhodes et al. (2002) for discussions. For example, activities of enzymes extracted from several mangrove algae were inhibited with increasing NaCl up to 600 mM. In contrast, when combined with equimolar concentrations of a mixture of several compatible solutes, mannitol, sorbitol, and a heteroside (a dimer of a hexose and glycerol), enzyme function was not inhibited (Karsten et al., 1996). Compatible solutes are thought to function by means of preferential exclusion, somehow stabilizing and ordering water molecules in the hydration shell surrounding macromolecules. Ions are prevented from penetrating the shell so that protein and solute do not come into direct contact (Timasheff, 1993). Compatible solutes represent a comparatively easy approach evolutionarily because tolerance only requires synthesis of one compound (through one biosynthetic pathway or modification of an existing pathway).

This approach is by no means uncommon. All organisms tolerate abiotic stress to some degree by accumulating "compatible" solutes (Rhodes et al., 2002). Thus, introduction of gene(s) for compatible solute biosynthesis with accumulation in the right compartment(s) could enhance dehydration and salt stress tolerance. There are a numerous candidates (Table 1) and now many lines of evidence supporting a beneficial effect of increased compatible solute production. Some of these solutes are primary photosynthetic products. Sucrose, for example, is present in all higher plants, sorbitol is ubiquitous in Rosaceous pome and stone fruits, and mannitol is found in 70 higher plant families (Loescher and Everard, 2000). Salinity often affects synthesis and accumulation of many of these compounds. For example, root-zone salinity altered raffinose oligosaccharide metabolism and transport in *Coleus* (Gilbert et al., 1997). In salt-stressed celery, mannitol becomes the predominant photosynthetic product (Everard et al., 1994). Sorbitol accumulates to high levels in the halophyte, *Plantago maritima*, up to 300 mmol·g⁻¹ dry wt in shoots and 225 mmol in roots) under saline (400 mM NaCl) conditions (Ahmad et al., 1979). In the facultative halophyte ice plant (*Mesembryanthemum crystallinum*), a novel methyl transferase (Imt1) is induced by osmotic stress leading to the synthesis of ononitol from inositol

Table 1. Compatible solutes (osmoprotectants) found in higher plants.^a

Candidates (classes of compatible solutes)	Examples
Quaternary ammonium zwitterions	Glycine betaine
Amino acids	Particularly proline
Cyclic polyols	Inositol and its methylated derivatives ononitol, pinitol, quebrachitol
Non-reducing sugars	Sucrose, raffinose, stachyose, verbascose, trehalose
Acyclic polyols (sugar alcohols)	Sorbitol (glucitol), mannitol, adonitol, ribitol, perseitol, and galactitol

^aThe amino and ammonium compounds have no net charge at physiological pH and the cyclic and acyclic polyols and sugars are all non-reducing carbohydrates.

(Vernon and Bohnert, 1992). When a gene encoding Imt1 was introduced into tobacco, transformants appeared phenotypically normal, exhibited Imt1 enzyme activity, accumulated ononitol, and were salt-tolerant (Sheveleva et al., 1997). In transgenic tobacco engineered by introduction of a bacterial gene for mannitol 1-phosphate dehydrogenase, transgenic plants with mannitol had an increased ability to tolerate high salinity (Tarczynski et al. (1993), and later work (Shen et al., 1997) showed that there was increased resistance to oxidative stress when mannitol biosynthesis was specifically targeted to chloroplasts. In petunia, this gene improved chilling tolerance (Chiang et al., 2005). Zhifang and Loescher (2003) showed that *Arabidopsis* plants transgenic for the celery mannose 6-phosphate reductase (M6PR) were quite salt-tolerant, phenotypically normal, accumulated mannitol to levels nearly as high as sucrose, and at onset of flowering accumulated high levels of a mannitol-glucose dimer. Later work showed that these plants were also photosynthetically normal and able to maintain high rates even at substantial levels of salt stress (Sickler et al., 2007). More recently, direct competition experiments between each transgenic line and corresponding parental genotypes in field tests have shown that M6PR plants have equivalent or greater fitness than their wild-type parents (Bigelow et al., 2010).

Amino compounds, e.g., glycine betaine and proline, have long been known to be involved in some forms of abiotic stress tolerance (Ashraf and Foolad, 2007; Rhodes and Hanson, 1993), and there is clear evidence for their roles in salt tolerance. Plants transformed with various genes for metabolism of these compounds are often more tolerant, to salt, drought, or both, either by increasing biosynthesis (Hayashi et al., 1997; Holmstrom et al., 2000; Hong et al., 2000; Kishor et al., 1995; Nomura et al., 1995) or by decreasing degradation (Nanjo et al., 1999). Horticultural examples include petunia in which there was improved drought tolerance (Yamada et al., 2005) and potato with improved salt tolerance (Hmida-Sayari et al., 2005).

Whatever the gene or the compatible solute, compartmentation is critical. It is not likely that compatible solutes are uniformly distributed throughout the cell. This distribution question is rarely answered, but it is essential to the proposed function because there may be an insufficient quantity of the compatible solute to achieve the desired effect if not limited to the cytoplasm or specific organelles such as chloroplasts. As already shown, targeting mannitol biosynthesis to the chloroplast effectively increased resistance to oxidative stress (Shen et al., 1997). Keller and Matile (1989) showed that in celery, petiole parenchyma mannitol was predominantly stored in the vacuole (81%) with a lesser amount in the cytosol (19%), but cytosolic concentrations were calculated to reach 300 mM, adequate to balance high levels of salts in apoplast and vacuole because the cytosol represents a very small fraction of total cellular volume.

COMPATIBLE SOLUTES VERSUS OSMOPROTECTANTS

Compatible solutes are assumed to accumulate to high concentrations without interfering with normal metabolism, but protective effects are often seen at levels too low for significant osmotic effects. Thus, "osmoprotectant" functions also have been suggested, e.g., scavenging of free radicals (acting to quench effects of reactive oxygen species), stabilization of macromolecular and membrane structures, or activity as low-molecular-weight chaperones (Bohnert and Jensen, 1996). Recent studies, however, suggest other osmoprotectant mechanisms. A transcriptome analysis of the presence of the M6PR transgene in salt stress-tolerant *Arabidopsis* (Chan et al., 2011) showed that in addition to mannitol biosynthesis, there was also activation of the downstream ABA pathway by upregulation of ABA receptor genes (*PYL4*, *PYL5*, and *PYL6*) and downregulation of *PP2C* genes (*ABI1* and *ABI2*). In *M6PR* transgenic lines, there were also increases in transcripts related to redox levels and cell wall strengthening pathways. These data indicate that mannitol-enhanced stress tolerance is attributable at least in part to increased expression of a variety of stress inducible genes. Similarly, Chen and Murata (2011) have shown that low levels of glycine betaine, applied exogenously or generated by transgenes for glycine betaine biosynthesis, can induce the expression of certain stress-responsive genes, including those for enzymes that scavenge reactive oxygen species. Kathuria et al. (2009) showed that glycine betaine-induced water stress tolerance in rice is associated with upregulation of a number of stress-responsive genes. Overall, 165 genes were upregulated more than twofold, and of these, at least 50 genes are known to be involved in plant stress responses. Somewhat similar results were also seen with overexpression of the trehalose-6-phosphate phosphatase gene in rice (Ge et al., 2008). Whether such effects are common in plants transgenic for osmoprotectants remains to be determined. However, introducing a metabolite or metabolic pathways in a species in which such are normally absent might be expected to affect flux through other pathways (Eastmond and Graham, 2003).

Similarly, perturbing ion homeostasis should be expected to affect regulation and/or flux through other transporters and pathways linked to these systems. For example, insertional mutagenesis of the *AtNHX1* vacuolar antiporter (Sottosanto et al., 2004) resulted in changes in expression of genes involved in intracellular vesicular trafficking, protein targeting, and other cellular processes. When *AtHKT1;1* was expressed specifically in the root cortical and epidermal cells of *Arabidopsis*, the native *AtHKT1;1* gene responsible for Na^+ retrieval from the transpiration stream was also upregulated, and there was also a significant increase in expression of vacuolar pyrophosphatases (Plett et al., 2010).

OTHER FACTORS—REGULATORY DETERMINANTS, TRANSCRIPTION FACTORS, AND SIGNAL PATHWAY INTERMEDIATES

Although modulating osmolyte metabolism and transport proteins might not have been expected to result in significant changes in gene expression, a number of transgenes used for stress tolerance are transcription factors that up- or downregulate gene expression. Unfortunately, the molecular sensing, signaling, and response pathways that govern biotic and abiotic stress tolerance are not yet well understood in many plant species, although functional analyses in *Arabidopsis* of many genes for transcription factors have revealed a complex and overlapping hierarchy of signaling networks between many different stresses (Bressan et al., 2008; Shinozaki et al., 2003; Witcombe et al., 2008). Much of this work has been carried out in *Arabidopsis* with the SOS signal pathway associated with salt tolerance (Bressan et al., 2008) or the dehydration-responsive element binding (DREB)/CBF (C-repeat binding factors) transcriptional activation factors (Gilmour et al., 2004; Shinozaki et al., 2003). There are now numerous examples of stress-tolerant plants transgenic for DREB/CBF with either constitutive promoters (often resulting in dwarfing) or under control of an *Arabidopsis* stress-inducible promoter (to reduce negative effects) (e.g., Achard et al., 2008; Gilmour et al., 2004; Haake et al., 2002; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Pino et al., 2007).

Regulatory intermediates that modulate plant salt stress responses include SOS3 (Ca²⁺-binding protein), SOS2 kinase, Ca²⁺-dependent protein kinases, and mitogen-activated protein kinases (Bressan et al., 2008). Other signal intermediates have been implicated in the response to salt, many through ectopic expression in transgenic plants. For example, the basic Leu zipper motif, MYB and MYC, AP2 family, PHD type, and zinc finger transcription factors, including rd22BP1 (MYC), AtMYB2 (MYB), and ALFIN1 (zinc finger), all interact with promoters of osmotic-regulated genes (Abe et al., 2003; Bhatnagar-Mathur et al., 2008; Gao et al., 2007; Kant et al., 2008; Oh et al., 2009; Shin et al., 2011; Winicov, 2000; Yang et al., 2011; Zhang et al., 2011; Zhu, 2002). Other examples include constitutive expression of CBF/DREB transcription factors in *Arabidopsis*, which leads increased expression of the CBF/DREB regulon genes, including LEA, dehydrin, antifreeze, and galactinol synthesis-related genes as well as factors involved in signal transduction and gene regulation (Fowler and Thomashow, 2002; James et al., 2008; Maruyama et al., 2004; Pino et al., 2007; Seki et al., 2001; Vogel et al., 2005; Zhang et al., 2004).

OTHER OPTIONS—PLANT HORMONES AND STRESS SENSORS

It is also now clear that the hormone ABA accumulates in plant tissues in response to

biotic, abiotic, and especially osmotic-based stresses, including salt stress (Harb et al., 2010; Himmelbach et al., 2003; Zhu, 2002). ABA is also a major component of plant stress response signaling networks (the *AREB/ABF* and *Myc/Myb* regulons that are involved in abiotic stress responses), and the ABA receptor family appears to be highly conserved in crop species, which suggests opportunities for manipulating stress tolerance in crops (Klingler et al., 2010). Consequently, there are now a number of approaches to stress tolerance that modulate ABA sensitivities (Li et al., 2010a; Ren et al., 2010; Saavedra et al., 2010; Santiago et al., 2009; Yang et al., 2010), regulation (Park et al., 2009), or biosynthesis (Ko et al., 2006), and the results have demonstrated improved salt and/or drought tolerance when expressed in transgenic plants.

Another hormone option is cytokinin biosynthesis. Upregulation or overexpression of *P-SARK::IPT*, a key step in cytokinin metabolism, results in delayed leaf senescence with a significant improvement in drought tolerance (Rivero et al., 2007). The mechanism relates to drought often stimulating senescence and abscission and with a cytokinin-mediated delay in senescence photosynthetic capacity is maintained (Rivero et al., 2010). Although there is as yet no evidence that this specific mechanism translates into salinity tolerance, there is clear evidence that cytokinins are involved in the salinity response (de la Pena et al., 2008; Mason et al., 2010; Tran et al., 2007).

Another approach may be identification of stress sensors (as the primary step in a plant's response). Putative sensors have been identified (Mikolajczyk et al., 2000; Urao et al., 1999), and there are numerous possibilities, e.g., physical changes in membrane proteins and kinases (Seo et al., 2010), release of metabolites like ATP (Baena-González and Sheen, 2008), or accumulation of reactive oxygen species activating various metabolic or signaling pathways (Miller et al., 2009). However, their specific functions remain elusive and analyses are complicated by the numbers and interactions of potential candidates and the possibility that there may be substantial redundancies (Mittler and Blumwald, 2010).

OTHER ISSUES—THE HORTICULTURAL PERSPECTIVE

Despite the evidence, particularly in model species, for non-traditional (transgenic) alternatives to classical plant breeding to achieve salinity tolerance, there are few examples of horticultural crops transformed to be salt-tolerant, even fewer are in field testing, and none are in production. Indeed, there is little evidence that breeding for salt tolerance is a priority for any horticultural crop. This may reflect higher returns that justify investments in more sophisticated irrigation and drainage systems that avoid salt stresses, or it may also represent the limited resources available for improvement of the so-called minor crops. There are also other problems. Although there has been substantial progress in plant regeneration and

transformation, creating transgenic plants is not yet routine for many crop plants (Barampuram and Zhang, 2011), and growers may also be reluctant to adopt transgenic crops that may encounter consumer acceptance and regulatory problems. Nonetheless, salinity remains a major threat to the sustainable irrigation required to meet the food demands of increasing populations and economic development (Flowers, 2004). Consequently, although progress might be slow, and as Munns (2009) has indicated, with the natural diversity that exists, and the current consumer acceptance issues, consideration could be given to using the substantial progress made in genes identified as markers for naturally occurring diversity or linked molecular markers to physiological traits. Indeed, there may be a relatively small number of QTL governing the complex physiological processes involved in the combination of drought stress and ion toxicities that comprise salinity tolerance. Uses of molecular (microsatellite) markers and QTL mapping are progressing rapidly, the techniques are relatively inexpensive, and they do not require extensive phenotypic screening (with the problems inherent in establishing reproducible levels of drought and salt stress). Given the extraordinary progress in sequencing (<http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi>), apparent similarities between species in signaling (Mochida et al., 2010), and the syntenic relationships between species, these can increasingly be exploited for comparative genomics analyses (Bennetzen, 2002) and dramatically improve the efficiencies in genetic mapping and ultimately crop improvement.

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