Bhumi Nath Tripathi · Maria Müller
Editors

Stress Responses in Plants

Mechanisms of Toxicity and Tolerance

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Chapter 1
Salt Stress in Higher Plants: Mechanisms of Toxicity and Defensive Responses

Anabella Fernanda Lodeyro and Néstor Carrillo

Abstract Soil salinity is a major constraint to crop performance. The main contributors to salt toxicity at a global scale are Na^+ and Cl^- ions which affect up to 50 % of irrigated soils. Effects of salt exposure occur at the organismic, cellular, and molecular levels and are pleiotropic, involving (1) osmotic and water deficit syndromes, (2) specific Na^+ and Cl^- inhibitions, (3) nutritional imbalance, and (4) oxidative stress. We review herein the responses elicited by salt-stressed plants to face all these challenges. With the only exception of halobacteria, all other organisms are not halotolerant at the molecular level. Instead, they have developed strategies to keep salts out of the cell. Then, induction of systems for salt extrusion to the rhizosphere and salt compartmentation into the vacuole play key roles in salt tolerance, aided by the synthesis and accumulation of compatible osmolytes and of antioxidant enzymes and metabolites. Expression of these effector genes is modulated by a complex network of salt-responsive transcription factors and signaling molecules. We discuss the progress made towards increasing salt tolerance in crops by engineering genes whose products operate at all these stages, from sensing and regulation to effector proteins, and identify key open questions that remain to be addressed.

Keywords Salt tolerance • Oxidative stress • Nutritional imbalance • Osmotic adjustment • Ion toxicity

1.1 Introduction

Earth is a predominantly salty planet, with most of its water containing ~600 mM NaCl. About 7 % of the firm land, 20 % of the cultivated land, and nearly half of the irrigated land are affected by high salt contents (Zhu 2001, 2002, 2003). The threats of salinity are more obvious in arid and semiarid regions where limited rainfall, high evapotranspiration, and extreme temperatures associated with poor water and

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soil management are the main contributing factors (de Azevedo Neto et al. 2006). While virtually all salts may have deleterious effects on plant welfare, the term salt stress usually refers to the consequences of abnormal accumulation of Na^+ and Cl^- ions, since this is by far the most extended environmental hardship related to salinity.

Salinization might occur by natural causes, primarily by capillary water level elevation and subsequent evaporation of saline groundwater. However, the increase of salinization at a global scale is largely due to human intervention, particularly in arid regions. Irrigation practices lead to groundwater level elevation and a subsequent increase in evaporation. Salts not only cause direct damage on plants but also provoke secondary negative effects, such as increase of the soil pH. Most plants do not grow well at high pH due to iron starvation. Iron is found in nature mostly as ferric oxides that are sparingly soluble, indicating that the main problem of Fe acquisition is not of abundance but of bioavailability (Curie and Briat 2003). The solubility product of ferric oxides decreases three orders of magnitude for each pH unit raise, which represents a major deterrent for agriculture in alkaline, calcareous soils that cover more than one third of the planet's cultivable land (Morel and Price 2003).

Salt stress affects plant physiology at whole plant as well as cellular and molecular levels and at all stages of development from germination to senescence (Hasegawa et al. 2000; Muranaka et al. 2002; Murphy et al. 2003; Ranjbarfordoei et al. 2002). Reported effects include changes in growth rate, ion toxicity, mineral limitation, membrane instability, photosynthesis, and increased respiration (Ashraf and Shahbaz 2003; Hasegawa et al. 2000; Munns 2002). Buildup of high amounts of salts in the leaf apoplast leads to dehydration and turgor loss (Marschner 1995), whereas salt accumulation in the cytosol and organelles results in inhibition of enzymes and metabolic pathways, including photosynthesis (de Lacerda et al. 2003). The reduction of plant growth and biomass accumulation under saline conditions has been reported in several important crops (Tejera et al. 2006).

To facilitate analysis, the unfavorable effects of salt stress are classified in four major groups: (1) osmotically induced water stress, (2) specific salt toxicity inhibiting enzymes and metabolic pathways, (3) nutrient ion imbalance due to high levels of Na^+ and Cl^- competing with the uptake of other essential ions, and (4) increased production of reactive oxygen species (ROS) which damage all types of macromolecules. These different modes of action will be briefly described in the forthcoming sections, followed by a survey of the responses elicited by salt-stressed plants, their regulation, and the use of the knowledge gathered in the last decades to design salt-tolerant plants. Several reviews on salt toxicity, sensing, and tolerance have been published in recent years (Bose et al. 2014; Deinlein et al. 2014; Golldack et al. 2011; Huang et al. 2012; Maathuis 2014). The reader is referred to them for a more extensive treatment of these subjects.

1.2 Osmotic Adjustment and Water Deficit

Salt accumulation in soils reduces the plant's ability to take up water from the rhizosphere, and this leads to water limitation and growth reduction. This process is the first to occur in a salt-stressed plant and is formally analogous to a water-deficit stress. Indeed, some cellular and metabolic processes involved in osmotic responses to salinity are common to drought, including stomatal closure (see below). The rates at which new leaves are produced depend largely on the water potential of the soil solution, in the same way as for a drought-stressed plant. At this stage, salts themselves do not build up in the growing tissues at concentrations that could inhibit growth, as the rapidly elongating cells can accommodate the salt that arrives in the xylem within their expanding vacuoles (Munns 2005). Then, reductions in the rate of leaf and root growth are due to water stress rather than a salt-specific effect (Munns 2002), since Na^+ and Cl^- are usually below toxic concentrations in the growing cells. For example, in wheat exposed to 120 mM NaCl, Na^+ in the growing tissues of leaves was at most 20 mM and only 10 mM in the rapidly expanding zones (Hu et al. 2005). Results of experimental manipulation of shoot water relations suggest that hormonal signals, probably induced by the osmotic effect of the salt on the roots, are controlling the rate of cell elongation (Munns et al. 1999). Inhibition of plant growth due to salinity largely depends on the severity of the stress. Mild osmotic challenges rapidly lead to growth inhibition of leaves and stems, whereas roots may continue to elongate (Hsiao and Xu 2000). The degree of growth inhibition due to this osmotic stress also depends on the time scale of the response for the particular tissue and species and on whether the stress treatments are imposed abruptly or gradually (Munns et al. 1999).

1.3 Specific Ion Toxicity

As salts are taken up by a plant, they tend to concentrate in the old leaves. However, continued transport into transpiring leaves over a long period of time eventually exceeds the ability of the cells to exclude salts from the cell or to compartmentalize them in the vacuole (see below). Salts then would build up in the cytosol and inhibit enzyme activity. Alternatively, they might accumulate in the cell walls and dehydrate the cell (Munns 2005). These specific effects of ions such as Na^+ and Cl^- follow the water and osmotic stresses that initiate the salinity syndrome.

Under normal growth conditions, root cytosolic Na^+ concentrations are probably in the order of 10–30 mM (Tester and Davenport 2003). Leaf Na^+ cytosolic concentrations are considered to be in the same range (Jones and Gorham 2002). Roots must exclude most of the Na^+ and Cl^- dissolved in the soil solution, or the salt concentration in the shoot will gradually increase to toxic levels. Plants transpire about 50 times more water than they retain in their leaves (Munns 2005).

The inhibitory concentrations of Na^+ vary depending on the reaction or metabolism, whereas the concentrations at which Cl^- becomes toxic are less defined. Metabolic routes affected include photosynthesis, nitrogen assimilation via nitrate and nitrite reductases, protein translation, and malate metabolism (Parida and Das 2005). The increase of Na^+ levels inside plant tissues also has toxic effects on seed germination, mainly by affecting the plant water relations and through displacement of Ca^{2+} by Na^+ from critical cell wall binding sites, which could disrupt cell wall synthesis and hence inhibit early plant growth (Xue et al. 2004).

1.4 Nutritional Imbalance

Ionic imbalance occurs in the cells due to excessive accumulation of Na^+ and Cl^- which reduces uptake of other mineral nutrients, such as K^+, Ca^{2+}, and Mn^{2+} (Karimi et al. 2005). Sodium and potassium are imported into the cell using the same suite of transporters, and the cations compete with each other (Greenway and Munns 1980). Excess Na^+ therefore inhibits K^+ uptake, leading to the appearance of symptoms of K^+ deficiency. Many central enzymes and metabolic routes require K^+ to acquire high specific activities compatible with life and development (Booth and Beardall 1991). This cation is also necessary for osmoregulation and protein synthesis, for the preservation of cell turgor and for optimal photosynthetic activity (Ashraf 2004; Freitas et al. 2001). Both K^+ and Ca^{2+} are required to maintain the integrity and functioning of cell membranes (de Lacerda et al. 2003; Munns 2002; Wei et al. 2003). Potassium deficiency initially leads to chlorosis and then necrosis (Gopa and Dube 2003).

The maintenance of calcium acquisition and transport under salt stress is also an important determinant of salinity tolerance (Soussi et al. 2001). Salt stress decreases the Ca^{2+}/Na^+ ratio in the root zone, which affects membrane properties due to displacement of membrane-associated Ca^{2+} by Na^+, leading to dissolution of membrane integrity and loss of selectivity (Kinraide 1998). Externally supplied Ca^{2+} has been shown to ameliorate the adverse effects of NaCl on plants by competition with Na^+, by increasing K^+/Na^+ ratio and by osmotic adjustment, through the enhancement of compatible organic solutes accumulation (Girija et al. 2002; Hasegawa et al. 2000).

Ca^{2+} also plays a critical role in the signaling network of plant cells. Extracellular stress signals can be perceived by the membrane receptors, which activate a large and complex signaling cascade, including the generation of second messengers such as Ca^{2+}. This increase in cytosolic Ca^{2+} concentration primes the signaling pathways for stress tolerance (Mahajan and Tuteja 2005; Tuteja and Mahajan 2007). Moreover, Ca^{2+}-binding proteins (calcineurin B-like proteins, CBLs) provide an additional level of regulation in Ca^{2+} signaling, initiating a phosphorylation/dephosphorylation cascade leading to regulation of gene expression and resulting in the expression of multiple responsive effector genes.

1.5 Oxidative Stress

Exposure of plants to salt stress can upregulate the production of ROS such as $O_2^{\bullet -}$ (superoxide radical), H_2O_2 (hydrogen peroxide), $^1O_2$ (singlet oxygen), and $^{\bullet}OH$ (hydroxyl radical). Excess ROS causes phytotoxic reactions including lipid peroxidation, protein degradation, and DNA mutation (Abogadallah 2010; McCord 2000; Pitzschke et al. 2006; Vinocur and Altman 2005; Wang et al. 2003). In plant cells, ROS are generated in the cytosol, chloroplasts, mitochondria, and the apoplastic space (Bowler and Fluhr 2000; Jacoby et al. 2011; Mittler et al. 2004; Mittler et al. 2011).

The main source of ROS in illuminated plants is the photosynthetic electron transport chain (PETC) of leaf chloroplasts. Salt-induced stomatal closure due to the water deficit and osmotic components of the salinity stress cause a decrease in CO_2 concentration inside chloroplasts leading to knockdown of the Calvin cycle by substrate limitation (Miller et al. 2010). NADPH is continuously produced at the thylakoids, but its oxidation in the regenerative stage of the Calvin cycle is blocked (Apel and Hirt 2004). Under these conditions, the PETC becomes over-reduced, and the propensity of O_2 to subtract electrons from the chain is expected to increase, leading to runaway ROS propagation, mostly $O_2^{\bullet -}$ and H_2O_2 (Foyer and Noctor 2000). In turn, ROS buildup damages the D1 protein of photosystem II causing photoinhibition. Stress-enhanced photorespiration in peroxisomes and misfunction of the respiratory chain of mitochondria also contribute to H_2O_2 accumulation (Miller et al. 2010). Major targets of $O_2^{\bullet -}$ toxicity are the iron-sulfur clusters of dehydratases and electron transfer proteins (Imlay 2006), whereas H_2O_2 may inactivate enzymes by oxidizing their thiol groups (Gill and Tuteja 2010). Toxic effects of H_2O_2 are enhanced by reaction with metal reductants, most conspicuously Fe^{2+}, to form the highly reactive hydroxyl radical, which is able to react with virtually any biological molecule (Halliwell and Gutteridge 1999).

In addition to these sources of salt-driven oxidative stress caused by misrouting of reducing equivalents from key redox pathways, ROS are also produced in the apoplast by a multigenic family of membrane-bound NADPH oxidases (Mittler et al. 2004). Given the many negative effects of ROS, it seemed at first odd that exposure to salinity led to induction of these enzymes, resulting in direct H_2O_2 propagation under conditions in which antioxidant defenses are activated to cope with ROS buildup. However, subsequent studies have shown that ROS can also play a key role in plants as signal transduction molecules involved in mediating responses to pathogen infection, environmental stresses, programmed cell death, and developmental stimuli (Mittler et al. 2004; Torres and Dangl 2005). In a phylogenomic study, Mittler et al. (2011) observed that the basic lot of antioxidants and scavenging enzymes were already present in the algal precursors of higher plants, while apoplastic NADPH oxidase activity is a newcomer to the plant kingdom in evolutionary terms, only found in vascular plants. These results suggest that photosynthetic eukaryotes learned first how to detoxify ROS and only later to use them as signaling molecules.

1.6 Plant Responses to Salt Stress

As indicated, salt stress affects plant physiology at almost all growth stages. Salt tolerance at these various developmental conditions varies widely from species to species. Plants that can survive and reproduce on high concentrations of salt in the rhizosphere are called halophytes. Depending on their salt-tolerant capacity, halophytes are either obligate or facultative, the latter ones displaying broader physiological resources that allow them to thrive in both saline and non-saline environments (Parida and Das 2005). Salt-sensitive species are termed glycophytes. Nearly all crops are glycophytes (Ashraf 2004).

With the exception of a few halophytic bacteria, there are no intrinsic salt-resistant enzymes or metabolisms. Tolerance is achieved by keeping salt out of the cell or into the vacuole and by combatting the damaging consequences of the stress situation. In halophytes, these mechanisms are more efficient than in glycophytes. For example, Jones and Gorham (2002) reported that the higher salt tolerance of *Agropyron junceum* with respect to *Agropyron intermedium* was related to more efficient exclusion of both Na^+ and Cl^-. In another study, Carden et al. (2003) found that a salt-tolerant barley variety maintained a tenfold lower cytosolic Na^+ in the root cortical cells than a more sensitive one. It is well established that most of the damage undergone by salt-exposed plants results from accumulation of Na^+ in shoots, inhibiting key metabolic processes such as protein synthesis and photosynthesis (Munns 2005). Thus, in most halophytes, Na^+ retention in the root is a general trend and hence an important component of salt tolerance (Ashraf 2004).

1.7 Ion Homeostasis

The maintenance of a high cytosolic K^+/Na^+ ratio is critical for salt tolerance (Glenn et al. 1999), and plants have evolved two main types of mechanisms to achieve these goals, those preventing the entry of Na^+ into the plant and those minimizing the concentration of Na^+ in the cytoplasm: extrusion and compartmentation (Fig. 1.1). In *Arabidopsis*, Na^+ influx is controlled by AtHKT1, a low-affinity Na^+ transporter (Rus et al. 2001; Uozumi et al. 2000). The knockout mutant *hkt1* from *Arabidopsis* suppressed Na^+ accumulation and sodium hypersensitivity (Rus et al. 2001), suggesting that AtHKT1 is a salt tolerance determinant. On the other hand, Na^+ efflux is controlled by Salt Overly Sensitive 1 (SOS1), a plasma membrane Na^+/H^+ antiporter (Shi et al. 2003), which is powered by the operation of a H^+-ATPase (Blumwald et al. 2000). In addition to its role as an antiporter, SOS1 may act as a Na^+ sensor (Zhu 2003).

The compartmentation of Na^+ in vacuoles provides an efficient and cost-effective mechanism to prevent its toxic effects in the cytosol (Fig. 1.1). The transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter (*AtNHX1* in

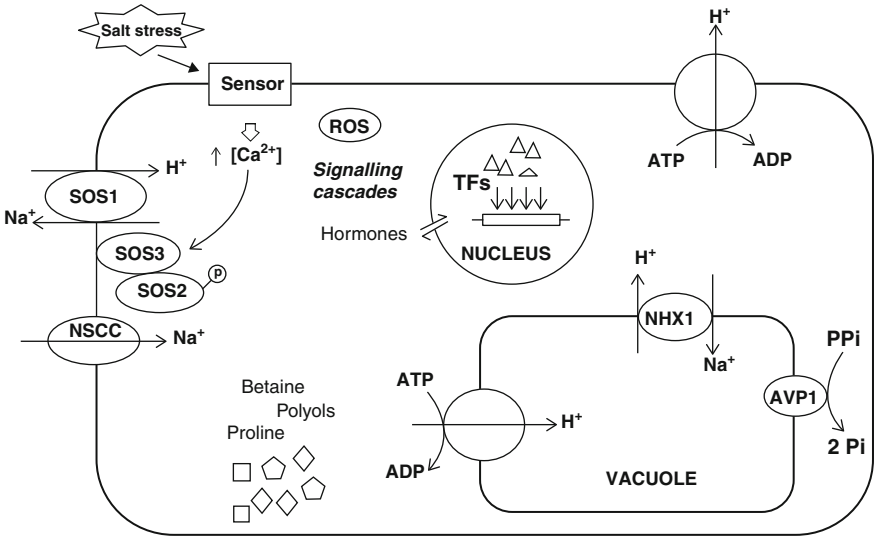

Fig. 1.1 Cellular responses to Na^+ toxicity. Na^+ is passively transported into the cell by nonselective cation channels (NSCCs), where it is sensed by an unidentified sensory system. Next, Ca^{2+}, ROS, and hormones activate signaling cascades which regulate the expression of multiple stress-related genes, resulting in the activation of cellular detoxification pathways. Two mechanisms operate to decrease cytosolic Na^+ concentration in the cell. One is to exclude Na^+ across the plasma membrane by the Na^+/H^+ exchanger SOS1. Another mechanism is to compartmentalize Na^+ into vacuoles by the vacuolar Na^+/H^+ antiporter, NHX1. Both transporter activities require a H^+ gradient across the membranes, which is generated by a plasma membrane H^+-ATPase or by vacuolar H^+-ATPase and H^+-pyrophosphatase (AVP1). Accumulation of osmoprotectants such as betaine, proline, and polyols is also induced by salt stress

Arabidopsis) that is driven by the electrochemical gradient of protons generated by vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-pyrophosphatase (Blumwald et al. 2000). Under salinization conditions, Na^+ influx into the cytosol could take place through pathways that normally function in the uptake of K^+, resulting in toxic levels of Na^+ as well as insufficient K^+ concentration for enzymatic reactions and osmotic adjustment. Three classes of low-affinity K^+ channels have been identified (Maathuis and Sanders 1995): these are K^+-inward rectifying channels (KIRCs), K^+-outward rectifying channels (KORCs), and voltage-independent cation channels (VICs). KORCs appear to be particularly important in mediating Na^+ influx into plant cells. These channels, which open during the depolarization of the plasma membrane, could mediate the efflux of K^+ and the influx of Na^+ ions under excess salt. Sodium competes with K^+ uptake through Na^+–K^+ co-transporters and may also block the K^+-specific transporters of root cells (Zhu 2003).

1.8 Synthesis of Compatible Solutes

The cellular response of organisms to both long- and short-term salinity stresses includes the synthesis and accumulation of a class of osmoprotective compounds known as compatible solutes (Fig. 1.1). These organic molecules can build up to high cellular concentrations without inhibiting metabolic processes. They comprise a varied assortment of chemicals including quaternary amines (glycinebetaine), amino acids (proline), soluble sugars such as trehalose, and sugar alcohols such as mannitol, sorbitol, and pinitol (Bose et al. 2014; Chinnusamy et al. 2006; Greenway and Munns 1980; Yeo 1998). These metabolites operate at various levels of the stress response. First, they provide osmotic compensation in the face of soil solutions containing high amounts of NaCl, attenuating the loss of water from the cell. For this reason, compatible solutes are known as osmolytes or osmoprotectants (Bohnert and Jensen 1996; Chen and Murata 2002). They also act as stabilizers of the quaternary structure of proteins and highly ordered states of membranes, preventing protein denaturation and membrane destabilization (Yancey et al. 1982). Finally, some of them (e.g., mannitol) serve as ROS scavengers, especially of those compounds which are too reactive to be detoxified enzymatically, such as the hydroxyl radical (Bose et al. 2014).

Genes involved in biosynthesis of compatible solutes are generally upregulated under salt stress, and concentrations of accumulated osmoprotectants correlate with osmotic stress tolerance (Zhu 2002). As could be expected, this response is largely shared with drought stress. Although enhanced synthesis and accumulation of compatible solutes under osmotic stress has been extensively documented, little is known about the signaling cascades that regulate their biosynthesis in higher plants.

The enhancement of glycinebetaine synthesis has received much attention (Rontein et al. 2002). In spinach and sugar beet, which naturally accumulate glycinebetaine, synthesis of this compound occurs in the chloroplast. The first oxidation to betaine aldehyde is catalyzed by choline monooxygenase and the subsequent oxidation to glycinebetaine by betaine aldehyde dehydrogenase (Rathinasabapathi 2000). In the soil actinobacterium *Arthrobacter globiformis*, the two oxidation steps are catalyzed by a single enzyme, choline oxidase (COD), encoded by the *codA locus* (Sakamoto and Murata 2000). Hayashi et al. (1997) used the *codA* gene of *A. globiformis* to engineer glycinebetaine synthesis in *Arabidopsis*. Tolerance to salinity during germination and seedling establishment was improved markedly in the transgenic lines. Huang et al. (2000) used *codA* from the related species *A. panescens* to transform *Arabidopsis*, *Brassica napus*, and tobacco. In this set of experiments, the COD protein was directed to the cytoplasm and not to the chloroplast. Improvements in tolerance to salinity, drought, and freezing were observed in some transgenic lines from all three species, but the tolerance was variable. The results confirmed that the protection by glycinebetaine is not only osmotic but also as an ROS scavenger. The level of glycinebetaine production in transgenic plants could be limited by the availability of choline, and a

dramatic increase in glycinebetaine contents in *Arabidopsis* was achieved when the growth medium was supplemented with choline (Huang et al. 2000).

1.9 Antioxidant Protection

Stress-induced production of ROS causes oxidative damage to many different cellular components including lipids, proteins, and nucleic acids (Miller et al. 2010), and different reports have shown that amelioration of the deleterious effects of stress-induced ROS could provide enhanced plant resistance to salinity (Ashraf and Harris 2004; Bose et al. 2014; Reguera et al. 2012; Roy et al. 2014). Plants use antioxidants such as reduced glutathione (GSH) and ascorbate (ASC), as well as enzymes specifically involved in ROS detoxification, including superoxide dismutases (SOD), catalases (CAT), glutathione *S*-transferases (GST), glutathione peroxidases (GPX), and ascorbate peroxidase (APX). It has been shown that expression of enzymes responsible for ROS scavenging and synthesis of antioxidants are induced by salt and other sources of environmental stress (Tester and Davenport 2003; Zhu 2001), supporting the notion that containment of cellular ROS buildup represents a major contribution to salinity tolerance and that overexpression of these genes is a promising strategy to develop salt-tolerant lines (see below). Ruiz and Blumwald (2002) investigated the enzymatic pathways leading to GSH synthesis during the response to salt stress of wild-type (WT) and salt-tolerant *B. napus* plants overexpressing a vacuolar Na^+/H^+ antiporter (Zhang et al. 2001). WT plants showed a marked increase in the activity of enzymes associated with cysteine synthesis (the crucial step for assimilation of reduced sulfur into organic compounds such as GSH), resulting in a significant increase in GSH content. On the other hand, these activities did not change with salt stress in the transgenic salt-tolerant plants, and their GSH levels were not modified. These results showed that salt stress induced an increase in the assimilation of sulfur and the biosynthesis of cysteine and GSH in order to mitigate salt-induced oxidative stress.

1.10 Regulation of the Responses

Transcription factors (TFs) are key regulators in salt stress responses, linking sensory pathways to tolerance (Figs. 1.1 and 1.2). Families of TFs are usually classified based on the nature of their DNA binding sites. The most relevant TF families are the basic leucine zipper (bZIP), WRKY, APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF), MYB, basic helix-loop-helix (bHLH), and NAC (Cui et al. 2013; Jiang and Deyholos 2009; Jiang et al. 2009; Kim et al. 2013; Tran et al. 2004; Yang et al. 2009). Genes belonging to different families are differentially expressed during stress, conferring adaptation and/or

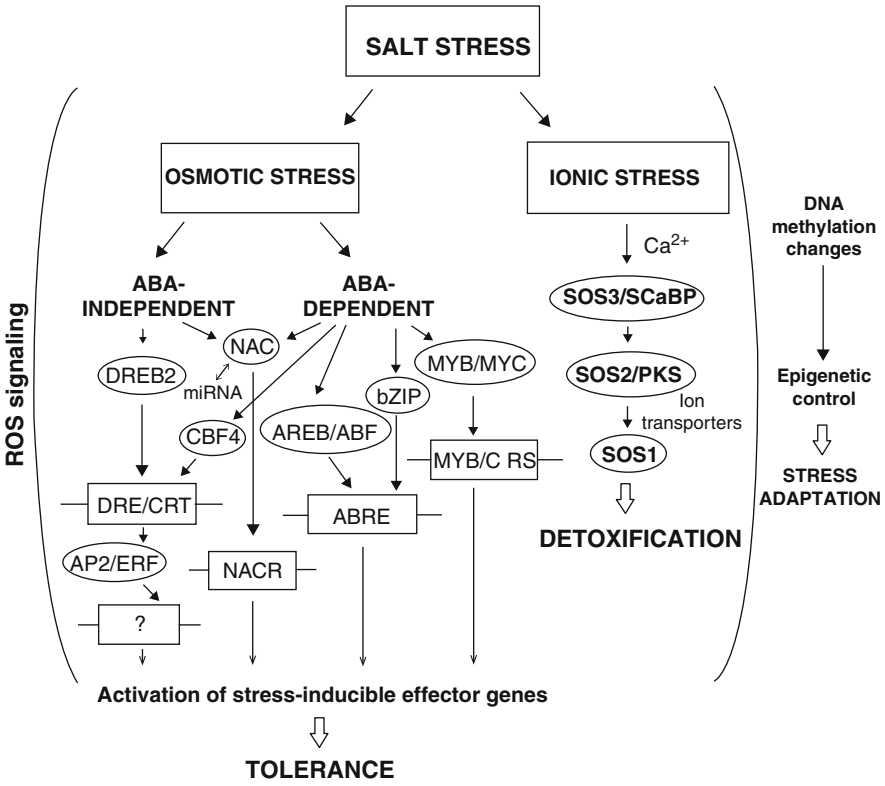

Fig. 1.2 Model of transcriptional signaling network involved in plant adaptation to salinity. Salt stress induces the activation of complex regulatory networks leading to the establishment of a defense response. ABA-dependent and ABA-independent pathways are involved in plant tolerance to salt stress. Osmotic stress signaling generated via salinity is mediated by transcription factors such as DREB2, bZIP, MYB, MYC, CBF4, and NAC, which interact with their corresponding recognition elements in the promoters of stress-dependent genes. Some transcription factors, including NAC and MYB, are themselves targets of drought- and salt-inducible miRNAs. ROS operate as signaling molecules within the regulatory networks. Salt stress detoxification mainly works through the SOS1/2/3 pathway keeping cellular ionic equilibrium. Fine-tuning of the response in plant environmental adaptation also involves epigenetic control of gene expression. Identities of the various components of this regulatory network are given in the text

tolerance to the adverse situation (Golldack et al. 2011). The expression levels of many effector genes, regulated by these TFs, may influence the magnitude of salt tolerance of plants (Deinlein et al. 2014; Golldack et al. 2011). For instance, genes encoding proteins related to ion uptake and osmolyte synthesis are upregulated by salinity, and transcriptional regulation of these stress response genes is mediated by dynamic changes in hormone levels (Geng et al. 2013).

The salt-responsive bZIP TF bZIP24 is induced by salt in *Arabidopsis thaliana* but suppressed in a halophytic *Arabidopsis* relative model species, acting as a key regulator of salt stress adaptation (Yang et al. 2009). RNAi-mediated repression of

this TF conferred increased salt tolerance to *Arabidopsis*, resulting in reduced Na^+ accumulation. Transcript analysis revealed that downstream potential target genes of AtbZIP24 function in osmotic adjustment, ion homeostasis, and plant development. Under normal growth conditions, transgenic *A. thaliana* plants in which bZIP24 levels were decreased by RNAi exhibited activation of stress-inducible genes: the Na^+ transporter HKT1, the Na^+/H^+ antiporter SOS1, the aquaporin PIP2.1, and a glutamine synthetase (Yang et al. 2009).

Endogenous levels of ABA increase in response to osmotic stress, and the phytohormone activates the expression of many genes via ABA-responsive elements (ABRE) in their promoter regions (Fig. 1.2). Transcription factors belonging to the ABRE-binding protein/ABRE-binding factor (AREB/ABF) family regulate the ABRE-mediated transcription of downstream target genes (Fujita et al. 2011). All members of this bZIP subfamily, AREB1, AREB2, and ABF3, are involved in drought stress in *Arabidopsis* via ABA signaling (Yoshida et al. 2010). In rice, a new bZIP TF, OsABF1, was isolated and characterized. It was shown to bind to ABREs and to be induced by anoxia, salinity, drought, oxidative stress, and ABA. In addition, the homozygous T-DNA insertional mutants *Osabf-1* and *Osabf-2* were more sensitive to drought and salt stress than WT plants (Hossain et al. 2010). Constitutive expression of the AREB ortholog TF from tomato (*Solanum lycopersicum*), SlAREB, increased tolerance to water deficit and high salinity in *Arabidopsis* and tomato plants, maintaining photosystem II and membrane integrities, and water content (Hsieh et al. 2010).

Functional specificity of bZIP factors in cellular transcriptional networks might be determined by specific homodimerizations and heterodimerizations which provide the resulting products with distinct DNA- and protein-binding properties, as well as conformational flexibility. Unfolded regions of the TF are responsible for transactivation. These regions contain protein recognition motifs and establish specific interactions with a wide range of protein targets that can be modulated by phosphorylation/dephosphorylation events (Miller 2009). The three TFs AREB1, AREB2, and ABF3 can form homodimers and heterodimers and interact with a SnRK2 protein kinase that regulates AREB1 (Yoshida et al. 2010). AtbZIP24 was targeted to the nucleus forming homodimers in response to salt stress (Yang et al. 2009). However, the salt-responsive TF bZIP1 forms heterodimers with other bZIP TFs (Weltmeier et al. 2009). Moreover, the TBP-associated factor AtTAF10 has a specific function in regulating accumulation of Na^+ and proline (Gao et al. 2006), this function overlapping those of bZIP24 (Yang et al. 2009).

The bZIP factors are located high in the hierarchical network of stress adaptation (Fig. 1.2), but other stress-related proteins play functional roles integrating the main pathways of environmental adaptation and regulating downstream sub-networks (Golldack et al. 2011). Members of the DREB/CBF subfamily of the AP2/ERF TFs play important roles in stress tolerance via ABA-dependent and ABA-independent pathways (Fig. 1.2), regulating a sub-transcriptome with more than 100 target genes (Shinozaki and Yamaguchi-Shinozaki 2000). Constitutive overexpression of DREB/CBF TF resulted in improved tolerance to drought, salt loading, and

freezing. However, the use of the strong constitutive 35S cauliflower mosaic virus (CaMV) promoter also caused severe growth retardation under normal growth conditions. In contrast, expression of DREB1A from a stress-inducible promoter had only marginal effects on plant growth while providing an even greater tolerance to stress conditions (Kasuga et al. 1999). The results illustrate the complexity of the stress adaptive network. Other example of multifunctional regulation is the R2R3-MYB TF AtMYB41 which is transcriptionally induced in response to ABA, drought, salinity, and cold (Lippold et al. 2009).

Transcription factors from the HD-Zip family contain a homeodomain (HD) associated to a leucine zipper (LZ). These TFs have been characterized as active participants in the adaptive response to several abiotic stresses. Their expression is regulated by drought, salt, and ABA (Ariel et al. 2007). Ectopic expression of the sunflower HaHB4 TF in *Arabidopsis* improved tolerance to drought and salinity, among other types of stresses (Cabello et al. 2007). In *Medicago truncatula*, *MtHB1* was identified as a salt stress-regulated gene (Ariel et al. 2007). Cabello and Chan (2012) have shown that the expression of the sunflower HaHB1 or its ortholog from *Arabidopsis* (AtHB13), as well as those of their putative targets, is upregulated by drought and salinity stresses. Transgenic plants overexpressing separately these genes exhibited increased tolerance to severe drought and salinity stresses, displaying cell membrane stabilization and higher chlorophyll contents (Cabello and Chan 2012).

The basic helix-loop-helix TF bHLH92 is induced in response to NaCl, dehydration, mannitol, and cold treatments. Root elongation of *bhlh92* mutants was more sensitive to mannitol and NaCl treatments compared to the WT, whereas overexpression of bHLH92 moderately increased the tolerance to NaCl and osmotic stresses. This TF regulates the expression of at least 19 downstream salt- and drought-responsive genes (Jiang et al. 2009).

WRKY factors modulate diverse plant processes, various biotic and abiotic stresses, and hormone-mediated pathways (Ramamoorthy et al. 2008). When WRKY25 and WRKY33 were overexpressed in *A. thaliana*, the transgenic plants showed increased tolerance to salt stress and ABA (Jiang and Deyholos 2009). Moreover, these TFs are also regulated by oxidative stress (Miller et al. 2010), with their target genes encoding proteins involved in ROS detoxification, such as peroxidases and GST (Jiang and Deyholos 2009). The expression of AREB1/ABF2 TF was affected in *wrky63* knockout mutants, demonstrating the involvement of WRKY factors in drought and salt adaptation via ABA-dependent pathways (Ren et al. 2010). Interestingly, WRKY factors are controlled and regulated by Zat proteins (TFIIIA-type Cys/His2f zinc finger proteins). The regulation of the soybean *GmWRKY54* gene, which confers drought and salt tolerance, by Zat10/SZT has been postulated by Zhou et al. (2008).

The NAC family of TFs is one of the largest ones and is only found in plants (Riechmann et al. 2000). NAC-type proteins play important roles in plant development (Souer et al. 1996; Xie et al. 2000) and have a key function in biotic and abiotic stress tolerance, including drought and salinity (Hegedus et al. 2003). The contribution of rice *NAC* genes to stress adaptation has been characterized;

OsNAC5 and *OsNAC6* are induced by ABA, drought, and salt stress (Rabbani et al. 2003). These factors bind directly to the promoter region of stress-inducible genes as *OsLEA3*, activating its transcription and promoting functional dimerization (Takasaki et al. 2010). Transgenic rice plants constitutively overexpressing the *OsNAC6* gene exhibited growth retardation and low reproductive yields, together with improved tolerance to dehydration and high-salt stresses (Nakashima et al. 2007). Overexpression of the *SNAC1* gene, belonging to this family, in rice plants resulted in stomatal closure, drought resistance, and improved salt tolerance under stressed field conditions (Hu et al. 2006). A recent study has shown that SNAC1-overexpressing cotton plants displayed improved tolerance to both drought and salt stresses under greenhouse conditions, enhancing root development and reducing transpiration rates (Liu et al. 2014). Another rice *NAC* gene, *SNAC2*, was identified as induced by drought, salinity, cold, wounding, and ABA, and its overexpression increased tolerance to salt, cold, and drought during rice seedling development. No common genes were found to be regulated by both SNAC1 and SNAC2 (Hu et al. 2008). Microarray studies demonstrated that two stress-responsive genes that were not affected in either *SNAC1* or *SNAC2* transgenic rice were upregulated in transgenic plants overexpressing OsNAC045, which display enhanced drought and salt tolerance (Zheng et al. 2009). These results indicate that different *NAC* genes have non-redundant functions, even though they are all involved in salt stress responses. Tran et al. (2007) have shown interaction of the drought-, salt- and ABA-inducible zinc finger protein ZFHD1 and an NAC factor.

Transcription factors also participate in adaptive responses to environmental stresses through microRNA (miRNA) pathways. Recently, an NAC-domain TF was identified as a target of miR164 in switchgrass (Matts et al. 2010). Moreover, SCL, MYB, and TCP TFs are targets of drought- and salt-inducible miRNAs as miR159, miR168, miR171, and miR396 (Liu et al. 2008b). In creeping bentgrass, salinity and drought stresses induce augmented expression of miR319, downregulating at least four putative target genes and a homolog of the rice *NAC*-domain gene, AsNAC60. Transgenic creeping bentgrass overexpressing the rice Os-miR319a exhibited enhanced salt tolerance (Zhou and Luo 2014). The regulation of stress-related targets through miRNAs may allow plants to fine-tune their responses to hormone and salt stress.

Epigenetic processes are becoming the new players in plant environmental adaptation. Chromatin structure might be modulated by DNA methylation and posttranslational histone modification (Kim et al. 2010), acetylation, phosphorylation, ubiquitination, biotinylation, and sumoylation (Chinnusamy et al. 2008). Plants growing in hostile habitats may carry memories of stress adaptation and transfer them epigenetically to the next generation (Fig. 1.2). The DNA of mangroves growing under saline conditions was hypomethylated, in contrast to populations from non-saline soils (Lira-Medeiros et al. 2010). In rice, salt stress modifies the expression of cytosine DNA methyltransferases (Sharma et al. 2009). In addition, Sokol et al. (2007) reported salinity-induced phosphorylation of histone H3 and acetylation of histone H4 in tobacco and *A. thaliana*, respectively. Studies

in stress responses related to epigenetics are slowly emerging, but it will be necessary to gain a more detailed knowledge on the specific mechanisms underlying epigenetic regulation under environmental stress to further improve salt tolerance in crop plants.

This brief enumeration, albeit partial, illustrates the bewildering complexity of the regulatory and signaling networks that are called into action when plants are exposed to salinity (Fig. 1.2) and their cross-talk with the responses invoked by other stresses such as drought and cold.

1.11 Strategies for Conferring Salt Tolerance Using Transgenic Plants

Two major approaches have been used to improve stress tolerance: (1) exploitation of natural genetic variations and (2) generation of transgenic plants with novel genes or altered expression levels of the existing ones. Zhang et al. (2004) and Zhu (2001, 2002) have reviewed signaling and transcriptional control in plants under salt stress, while Roy et al. (2014) discussed strategies to improve salinity tolerance of crops in an agronomical context. Since abiotic stress tolerance is multigenic in nature, the main trend has been to generate more tolerant transgenic plants by genetic transformation with multiple genes or with transcription factors. Many crop plants have been engineered using abiotic stress-related genes and have shown increased tolerance under laboratory conditions (Table 1.1, see also Agarwal et al. 2013). Genes whose products combat salt toxicity at various levels (ion homeostasis, synthesis of compatible solutes, and oxidative stress management) have been assayed to improve salt tolerance (Table 1.1). Many of these genes are induced during salinity, and therefore, salt induction has also been used as a criterion to identify tolerance-related traits, assuming that if expression of a gene is induced, it should be involved somehow in defense. In a different context, strategies for augmenting salt tolerance in glycophytes have been based on the expression of genes differentially regulated in salt-resistant and salt-sensitive cultivars. Finally, manipulation of stress-related TFs and signaling components offer the opportunity to modulate a suite of effector genes by a single intervention (Table 1.1).

An example of a protein involved in general processes that confers protection to salt stress is nucleolin. Nucleolin is involved in the assembly of ribosomal proteins with RNA (Didier and Klee 1992). Expression of nucleolin is reported to be regulated by drought, cold, and salinity stresses in an *Arabidopsis* microarray (Seki et al. 2002). Transgenic *Arabidopsis* plants overexpressing a rice nucleolin (OsNUC1) displayed higher relative growth rate, longer root length, and lower H_2O_2 accumulation under salt stress with respect to WT plants (Sripinyowanich et al. 2013). The pea helicase PDH45 was shown to be involved in salt tolerance by expression in transgenic tobacco (Sanan-Mishra et al. 2005) and rice (Sahoo

Table 1.1 Candidate gene families expressed in plants to improve salt tolerance

Transgene	Transgenic plant	Phenotype	References
Transcription factors			
OsDREB1	*Arabidopsis*	Salt tolerance	Zhang et al. (2009b)
	Rice	Salt, drought, and cold tolerance	Wang et al. (2008)
GmDREB2	*Arabidopsis*	Salt and dehydration tolerance	Chen et al. (2007)
GhDREB2	Wheat	Salt, drought, and cold tolerance	Gao et al. (2009)
OsDREB2A	Rice	Salt and dehydration tolerance	Mallikarjuna et al. (2011)
PgDREB2A	Tobacco	Salt and dehydration tolerance	Agarwal et al. (2010)
LcDREB3	*Arabidopsis*	Salt and drought tolerance	Xianjun et al. (2011)
MtCBF4	*Medicago truncatula*	Salt tolerance	Li et al. (2011a)
	Arabidopsis	Salt and drought tolerance	
AtMYB20	*Arabidopsis*	Salt tolerance	Cui et al. (2013)
TaMYB2A	*Arabidopsis*	Salt and drought tolerance	Mao et al. (2011)
CpMYB10	*Arabidopsis*	Salt and desiccation tolerance	Villalobos et al. (2004)
OsMYB3R	*Arabidopsis*	Salt, drought, and freezing tolerance	Dai et al. (2007)
GmMYB76	*Arabidopsis*	Salt and freezing tolerance	Liao et al. (2008)
AtMYB44	*Arabidopsis*	Salt and drought tolerance	Jung et al. (2008)
SlAIM1 (*MYB*)	Tomato	Salt and drought tolerance	Abuqamar et al. (2009)

(continued)

Table 1.1 (continued)

Transgene	Transgenic plant	Phenotype	References
GmERF3	Tobacco	Salt and dehydration tolerance	Zhang et al. (2009a)
OsNAC063	*Arabidopsis*	Salt and osmotic stress tolerance	Yokotani et al. (2009)
GmNAC11	*Arabidopsis*	Salt tolerance	Hao et al. (2011)
AhNAC2	*Arabidopsis*	Salt and drought tolerance	Liu et al. (2011b)
OsNAC5	*Arabidopsis* Rice	Salt and drought tolerance	Song et al. (2011)
OsSNAC2	Rice	Salt and cold stress tolerance	Hu et al. (2008)
OsNAC1	Rice	Salt tolerance	Hu et al. (2006)
DgNAC1	Tobacco	Salt tolerance	Liu et al. (2011a)
TaNAC69	Wheat	Inducible salt and drought tolerance	Xue et al. (2011)
Compatible solutes			
ScTPS1 (trehalose-6-phosphate synthase)	*Arabidopsis* Tomato	Salt tolerance	Miranda et al. (2007), Cortina and Culiáñez-Macià (2005)
ScTPS1-TPS2	Alfalfa	Salt tolerance	Suárez et al. (2009)
OsTPS1	Rice	Salt and drought tolerance	Li et al. (2011b)
otsA/*otsB* (*E. coli* trehalose-6-phosphate synthase/phosphatase)	Rice	Salt, drought, and cold tolerance	Garg et al. (2002)
mt1D (mannitol-1-phosphate dehydrogenase)	*Arabidopsis* Tobacco Wheat	Salt and dehydration tolerance	Thomas et al. (1995), Karakas et al. (1997), Abebe et al. (2003)
VaP5CS (Δ^1-pyrroline-5-carboxylate synthase)	Tobacco Rice Wheat	Salt tolerance	Kishor et al. (1995), Hong et al. (2000), Zhu et al. (1998), Sawahel and Hassan (2002)
AtP5CS	Potato	Salt tolerance	Hmida-Sayari et al. (2005)
codA (*A. globiformis* choline oxidase)	*Arabidopsis* Rice *Brassica juncea*	Salt tolerance	Hayashi et al. (1997), Sakamoto et al. (1998), Sakamoto and Murata (2000), Prasad et al. (2000)
betA (*E. coli* choline dehydrogenase)	Broccoli	Salt tolerance	Bhattacharya et al. (2004)

(continued)

Table 1.1 (continued)

Transgene	Transgenic plant	Phenotype	References
betA/betB (*E. coli* choline dehydrogenase/betaine aldehyde dehydrogenase)	Tobacco	Salt tolerance	Holmstrom et al. (2000)
AtBADH (betaine aldehyde dehydrogenase)	Wheat	Salt tolerance	Guo et al. (1999)
SoBADH	Tobacco	Salt tolerance	Yang et al. (2008), Fan et al. (2012)
	Sweet potato		
ROS detoxification			
AtAPX (ascorbate peroxidase)	Tobacco	Salt and drought tolerance	Badawi et al. (2004)
StAPX	Tobacco	Salt and osmotic stress tolerance	Sun et al. (2010)
PsAPX	Tomato	Salt tolerance	Wang et al. (2005)
OsAPX	*Arabidopsis*	Salt tolerance	Lu et al. (2007)
NtGST/GPX (glutathione *S*-transferase/glutathione peroxidase)	Tobacco	Salt tolerance and chilling	Roxas et al. (1997)
SsGST	*Arabidopsis*	Salt tolerance	Qi et al. (2010)
AmCu/ZnSOD (superoxide dismutase)	Rice	Salt and drought tolerance	Prashanth et al. (2008)
PsCu/ZnSOD/APX	Tobacco	Salt and osmotic stress tolerance	Lee et al. (2010)
AtMnSOD	*Arabidopsis*	Salt and cold tolerance	Wang et al. (2004)
OsDHAR (dehydroascorbate reductase)	*Arabidopsis*	Salt tolerance	Ushimaru et al. (2006)
AtMDAR (monoDHAR)	Tobacco	Salt, osmotic, and ozone stress tolerance	Eltayeb et al. (2007)
AmMDAR	Tobacco	Salt tolerance	Kavitha et al. (2010)
katE (*E. coli* catalase)	Rice	Salt tolerance	Moriwaki et al. (2008)
Ion transporters			
AtNHX1 (vacuolar Na^+/H^+ antiporter)	*Arabidopsis*	Salt tolerance	Apse et al. (1999), He et al. (2005), Xue et al. (2004), Zhang et al. (2001), Zhang and Blumwald (2001), Zhao et al. (2007), Chen et al. (2008), Liu et al. (2008a)
	Cotton		
	Tomato		
	Rapeseed		
	Tall fescue		
	Buckwheat		
	Sugar beet		
	Wheat		

(continued)

Table 1.1 (continued)

Transgene	Transgenic plant	Phenotype	References
SOD2 (yeast Na^+/H^+ antiporter)	*Arabidopsis*	Salt tolerance	Gao et al. (2003), Zhao et al. (2006)
	Rice		
AtSOS1 (plasma membrane Na^+/H^+ antiporter)	*Arabidopsis*	Salt tolerance	Shi et al. (2003), Yue et al. (2012)
	Tobacco		
SbSOS1	Tobacco	Salt tolerance	Yadav et al. (2012)
AtHKT1;1 (Na^+ transporter)	Rice	Salt tolerance	Plett et al. (2010)
HvHKT2;1 (K^+ transporter)	Barley	Salt tolerance	Mian et al. (2011)
AtAVP1 (vacuolar H^+ pyrophosphatase)	*Arabidopsis*	Salt tolerance	Gaxiola et al. (2001), Bao et al. (2009), Pasapula et al. (2011), Li et al. (2010)
	Alfalfa		
	Cotton		
	Creeping bentgrass		

et al. 2012). However, the exact mechanism of PDH45-mediated salinity stress tolerance is not well understood. Gill et al. (2013) have proposed that PDH45 coordinates the action of components of the antioxidant machinery in the transgenic plants. Recently, a gene encoding for a small RNA-binding protein (*S-RBP11*) was isolated as a salt-resistant activation tagging line. *Arabidopsis* plants overexpressing S-RBP11 showed increased tolerance to salt and oxidative stresses compared to WT plants (Lee et al. 2014).

A rational strategy to enhance salt tolerance is by manipulating the expression of effector genes, such as those involved in Na^+ extrusion of the cell or Na^+ import to the vacuole (Table 1.1). Salt-tolerant *B. napus* plants overexpressing the vacuolar Na^+/H^+ antiporter AtNHX1 from *A. thaliana* showed increased Na^+ accumulation in vacuoles and were able to grow in high saline concentration (Zhang et al. 2001). Comparative studies of salt-resistant and salt-sensitive cultivars of wheat have shown differential increase in TaNHX3, a vacuolar Na^+/H^+ antiporter. Expression of TaNHX3 in tobacco significantly enhanced salt tolerance, showing higher fresh and dry weights, contents of chlorophylls, carotenoids and soluble proteins, and increased antioxidant activities (Lu et al. 2014). Overexpression of NHX1 improved salt tolerance in *Arabidopsis* (Apse et al. 1999), tomato (Zhang and Blumwald 2001), maize (Yin et al. 2004), wheat (Xue et al. 2004), rice (Ohta et al. 2002), tobacco (Wu et al. 2004), and tall fescue plants (Tian et al. 2006). All these transformants were able to grow, flower, and set fruit in significantly higher salt concentration compared to their WT siblings.

Overexpression of AtSOS1 in *Arabidopsis* increased salt tolerance by limiting Na^+ accumulation in the xylem and stem (Shi et al. 2003), whereas transgenic

tobacco lines overexpressing AtSOS1 displayed better germination rates, lower chlorophyll loss, and less accumulation of Na^+ under salt treatments compared to WT plants (Yue et al. 2012). It has been recently reported that the expression of a truncated-hyperactive form of durum wheat TdSOS1 conferred significant ionic stress tolerance in *Arabidopsis*. In this context, the authors suggested that selection of hyperactive alleles of SOS1 may pave the way for obtaining salt-tolerant crops (Feki et al. 2014). Another interesting example is the expression of an *Arabidopsis* vacuolar H^+-pyrophosphatase gene (AVP1), which improved drought and salt tolerance in cotton, increasing proline contents and enhancing fiber yield under field conditions (Pasapula et al. 2011).

High salt concentrations lead to secondary stresses by enhancing the production of ROS which ultimately cause oxidative damage (Gill and Tuteja 2010; Munns and Tester 2008). ROS are toxic molecules, but also serve as mobile signals that regulate stress responses. Development of transgenic plants overexpressing one or more antioxidant enzymes is a common strategy used to obtain lines tolerant to salt stress in different species (Table 1.1). Transgenic tobacco plants overexpressing both GST and GPX showed improved seed germination and seedling growth under stress (Roxas et al. 1997). Also, tobacco transformants expressing a cytosolic APX from *Lycium chinense* (LmAPX) exhibited lower H_2O_2 accumulation, higher proline contents, and net photosynthetic rates under salt stress (Wu et al. 2014). Diaz-Vivancos et al. (2013) have reported increased tolerance to salt stress in plum by ectopic expression of cytosolic Cu/Zn SOD and APX. Transgenic plantlets exhibited higher contents of GSH and ASC and lower accumulation of H_2O_2 than the non-transformed control (Diaz-Vivancos et al. 2013). Synergistic effects were observed in cotton plants overexpressing a Cu/Zn SOD and CAT. Plants accumulating the two antioxidant enzymes in their chloroplasts exhibited the highest tolerance to salt stress compared with lines expressing the genes in the cytoplasm or with the single transformants (Luo et al. 2013).

As indicated, compatible solutes play important roles as osmoprotectants. Generally, manipulation of genes whose products are involved in the synthetic and degradative pathways of many osmolytes resulted in enhanced salt and drought tolerance (Table 1.1).

Initial strategies aimed at engineering higher concentrations of proline began with the overexpression of genes encoding the enzymes pyrroline-5-carboxylate synthetase (P5CS) and P5C reductase (P5CR), which catalyze the two steps between the substrate (glutamic acid) and the product (proline). P5CS overexpression in transgenic tobacco dramatically elevated the levels of proline (Kishor et al. 1995). However, there is strong evidence that free proline inhibits P5CS (Roosens et al. 1999). Hong et al. (2000) achieved a twofold increase of proline levels in tobacco plants by using a *P5CS* modified by site-directed mutagenesis. The procedure alleviated the feedback inhibition of P5CS activity by proline and resulted in improved germination and growth of seedlings under salt stress. Also, Nanjo et al. (1999) used antisense cDNA transformation to decrease proline dehydrogenase expression in order to increase free proline levels.

On the other hand, the introduction of the enzyme responsible for the synthesis of a proline precursor, Δ^1-pyrroline-carboxylate synthase, provides tolerance to salinity stress in a wide range of transgenic species: tobacco (Hong et al. 2000; Kishor et al. 1995), rice (Su and Wu 2004; Zhu et al. 1998), bread wheat (Sawahel and Hassan 2002), and potato (Hmida-Sayari et al. 2005).

Glycinebetaine is synthesized by a two-step process from choline with the intermediate betaine aldehyde. However, several crop plants could not synthesize betaine aldehyde (Rhodes and Hanson 1993). Genetically engineered tobacco plants synthetizing glycinebetaine in chloroplasts were obtained by introducing the betaine aldehyde dehydrogenase gene. The transgenic plants showed enhanced tolerance to salt stress by protecting photosynthesis (Yang et al. 2008). The gene encoding choline dehydrogenase from *Escherichia coli* has been expressed in *B. oleracea* (Bhattacharya et al. 2004) and maize (Quan et al. 2004), enhancing salt and drought tolerance. Moreover, rice plants expressing an inducible COD gene displayed greater salt tolerance due to increased production of glycinebetaine (Su et al. 2006).

The genes for trehalose synthesis from *E. coli*, *otsA*, and *otsB*, encoding trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively, were introduced in rice (Garg et al. 2002) and potato (Yeo et al. 2000), increasing tolerance to salt, drought, and cold stress in the transgenic plants. In a different approach, the gene encoding the trehalose-6-phosphate synthase from yeast was expressed in rice, tomato (Cortina and Culiáñez-Macià 2005), *Arabidopsis* (Miranda et al. 2007), and alfalfa (Suárez et al. 2009), imparting enhanced salt tolerance to these transgenic plants.

The accumulation of sugar alcohols is a widespread response to environmental stresses including salinity (Table 1.1). Salt tolerance of transgenic tobacco engineered to accumulate mannitol was first demonstrated by Tarczynski et al. (1993). Transgenic tobacco plants were engineered by introduction of the *E. coli* gene encoding mannitol-1-phosphate dehydrogenase. These plants synthesized and accumulated mannitol, increasing their ability to tolerate high salinity (Tarczynski et al. 1992, 1993). Similar results were obtained in *Arabidopsis* (Thomas et al. 1995) and wheat (Abebe et al. 2003). In line with a stress-protective role, overexpression of enzymes involved in the synthesis of inositol (L-myo-inositol-1-phosphate synthase and myo-inositol 1-phosphate phosphatase) from halotolerant plants increased cyclic polyols levels, resulting in salt stress tolerance in tobacco (Majee et al. 2004).

Other examples of genetic engineering directed to the accumulation of compatible solute include the transformation of tobacco cells with genes encoding enzymes for ectoine synthesis from the halophilic bacterium *Halomonas elongata* (Nakayama et al. 2000) and for sorbitol synthesis in *Plantago* (Pommerrenig et al. 2007).

Key transcription factors may regulate the expression of a range of salinity tolerance genes involved in several mechanisms, so it is reasonable to think that manipulation of TFs may result in the greatest effect on crop salinity tolerance with minor genetic modifications. However, the beneficial effects of TFs can be

counterbalanced by the introduction of yield penalties, especially under mild- or non-stress growth conditions. Examples of genetically modified plants using TFs are given in Table 1.1.

The discoveries made in the last few years on the mechanism of salt tolerance should be applied to crops to improve their performance. However, most of the transgenic plants studied are model plants, and stress tolerance has largely been evaluated under laboratory conditions. Salt tolerance must be assayed in the field and during periods of intense or prolonged stress resembling natural conditions. Fine-tuning of the expression of known candidates for stress tolerance genes obeying specific temporal and spatial patterns is essential to rule out negative effects on plant growth. The use of stress-inducible promoters is a good choice for developing stress tolerance while avoiding developmental penalties.

1.12 Conclusion

Environmental stress due to salinity is one of the most serious factors limiting the productivity of agricultural crops, most of which are sensitive to the presence of high concentrations of salts in the soil. About 50 % of irrigated agricultural land is adversely affected by salinity (Flowers and Yeo 1995). The problem of soil salinity is further aggravated through the use of poor quality water for irrigation and inadequate drainage. Soil type and environmental factors, such as vapor pressure deficit, radiation, and temperature, may also alter salt tolerance. The loss of farmable land to salinization is in direct conflict with the needs of the world population, projected to increase by 1.5 billion in the next 20 years (Blumwald and Grover 2006). Engineering crops that are resistant to salinity stress is critical for sustaining food production and achieving future food security. However, progress in breeding for salt-tolerant crops has been hampered by the lack of understanding of the molecular basis of salt tolerance and insufficient availability of genes whose products confer salt tolerance. Also, the evaluation of salt tolerance in transgenic lines has mostly been carried out using a limited number of seedlings or mature plants under laboratory and/or greenhouse conditions different from those which plants would naturally be exposed to in the field (Mittler 2006). The evaluation of field performance under salt stress is difficult because of the variability of salt levels under field conditions and the potential for interactions with other environmental factors, including soil fertility, temperature, light intensity, and water loss through transpiration (Daniells et al. 2001). The lack of success is also due in part to plant geneticists using constitutive promoters such as the CaMV 35S, ubiquitin, and actin promoters (Grover et al. 2003). In general, stress-induced or tissue-specific promoters result in better phenotypes than those obtained by expressing the same genes under control of a constitutive promoter (Kasuga et al. 1999; Zhu et al. 1998). There is a clear and urgent need to introduce these tolerance genes into crop plants, in addition to establishing gene stacking or gene pyramiding.

Although progress in increasing salt tolerance has been relatively slow, there are reasons for optimism. They include, among others, the development of molecular markers and gene tagging methodologies, the complete sequencing of plant genomes, the availability of forward genetics tools such as tilling, and the widespread use of microarray analysis. These powerful resources offer advantages and provide solutions to the complex and intriguing questions of salt resistance.

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