

Chapter 9

Salt Adaptation Mechanisms of Halophytes: Improvement of Salt Tolerance in Crop Plants

Rohit Joshi, Venkata Ramanarao Mangu, Renesh Bedre, Luis Sanchez, Whitney Pilcher, Hana Zandkarimi, and Niranjan Baisakh

Abstract Soil salinity is one of the most serious environmental factors that affect crop productivity worldwide. Inevitable global climate change leading to rise in sea water level would exacerbate degradation of irrigation systems and contamination of ground water resources, which render conventional agricultural practices impossible due to the sensitivity of most crops to salinity. Breeding for development of salt-tolerant crop plants has been a major challenge due to the complexity and multigenic control of salt tolerance traits. Halophytes are capable of surviving and thriving under salt at concentrations as high as 5 g/L, by maintaining negative water potential. Physiological and molecular studies have suggested that halophytes, unlike glycophytes, have evolved mechanisms, such as ion homeostasis through ion extrusion and compartmentalization, osmotic adjustments, and antioxidant production for adaptation to salinity. Employment of integrated approaches involving different omics tools would amplify our understanding of the biology of stress response networks in the halophytes. Translation of the knowledge and resources generated from halophyte relatives of crop plants through functional genomics will lead to the development of new breeds of crops that are suitable for saline agriculture.

Keywords Functional genomics • Crop plants • Halophyte • Salt tolerance • Smooth cordgrass

R. Joshi

Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, Delhi 110 067, India

V.R. Mangu • R. Bedre • L. Sanchez • W. Pilcher • H. Zandkarimi • N. Baisakh (✉)
School of Plant, Environmental, and Soil Sciences, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA
e-mail: nbaisakh@agcenter.lsu.edu

9.1 Introduction

World population is increasing continuously, while there is constant reduction in arable lands due to salinity of the soils (Kafi and Khan 2008). Soil salinity is one of the most serious environmental factors limiting the agricultural productivity and quality of crop plants worldwide (Flowers et al. 2010). No other toxic substance is known to restrict plant growth more than salt (Xiong and Zhu 2002). Salinity degrades agricultural landscape by natural and human interventions (Boesch et al. 1994; Rogers and McCarty 2000), which has been further enhanced by agricultural practices (Zhu 2001).

Significant percentages of arable land are affected by salinity, which renders conventional agriculture impractical because most field crops are salt sensitive. Thus, soil salinity poses a serious threat to global food security and sustainability. Approximately 1,000 million hectares of land is salt affected, which is over 7 % of the world's land area and 20 % of the world-cultivated area (Munns 2005; Jia et al. 2011). Salt-affected land cost over US\$12 billion per annum globally (Qadir et al. 2008). Irrigated land, despite being comparatively a small proportion of the total global agricultural area, produces one third of the total food (Munns and Tester 2008). Irrigation systems are particularly prone to saline soil; about 45–60 million ha of irrigated lands were damaged by salt, which is 20–25 % of the total irrigated area in the world (Glenn et al. 1999). Salt-affected land is increasing worldwide through deforestation and ground water irrigation, which raise the water table carrying dissolved salts to soil surface. Thus, vast regions of seacoast and inland lie barren because of inadequate or poor quality underground water available for irrigation (Gul et al. 2009). Salinity-induced crop damage is more acute in resource-poor third world countries mainly located in arid, semiarid, and coastal regions (Allakhverdiev et al. 2000; Munns 2002; Koca et al. 2007; Viégas et al. 2001).

One solution to tackle the salinity problem is to recycle the water near the site of production of salt-tolerant crops, thus reducing the amount of disposed water (Grieve and Suarez 1997). A limitation to this approach is low salt tolerance of agricultural crops and trees (Glenn et al. 1999). Conventional approaches using primary and secondary gene pools in crop plants have yielded salt-tolerant crops; the development has, however, been slow due to complexity and multigenic nature of the salt tolerance traits. On the other hand, reclaiming saline lands for agricultural crops is expensive, but they could be used to grow halophytes (Qureshi et al. 1991). Several halophytes, like salt marsh plants can flourish well in soils with poor drainage. Identifying and making use of desirable traits of suitable halophytic plants not only meet our requirement of salt-tolerant crops but also reduces pressure on arable lands (Khan and Weber 2006).

A salt-tolerant plant is able to maintain its growth under saline conditions relative to its growth under normal nonsaline conditions. High salt concentration disrupts homeostasis in plant water potential and ion distribution at cellular level, leading to degradation of biological molecules, growth arrest, and cell death (Munns and Tester 2008). To sustain future agricultural production, we must be able to develop salt-tolerant food and fiber plants other than salt-sensi-

tive glycophytes, which can successfully grow in salt-affected areas (Rozema and Flowers 2008).

Systems level understanding of plant biology, physiology, genetics, and biochemical approaches to overcome salinity stress responses is required, which can be used for engineering salt tolerance in crops. Genes that are regulated under salt stress can be identified either through transcriptomics (Kawasaki et al. 2001) or proteomics (Salekdeh et al. 2002). Several mechanisms are known to be operating in halophytes to adapt to soil salinity; it is therefore important to study these mechanisms in detail that will allow us for devising strategies for tailoring crops plants to survive and grow under salinity. This chapter provides information on physiological, biochemical, and molecular basis of salinity tolerance in halophytes, which can be used for the development of salt-tolerant crop plant.

9.2 Halophytes: Definition and Classification

Halophytes are considered to be remarkable plants that are much versatile in unrelated plant families during the divergent evolution of angiosperms and that tolerate salt at concentrations considered lethal to 99 % of other species (Trotta et al. 2012). Halophytes are known for several years; however, their definition still remains unclear. Flowers et al. (1986) defined halophytes as the plants with the ability to complete their life cycle at or above 200 mM NaCl, which is possibly encountered in the natural environment. Ayers and Wescott (1989) defined halophyte plants as those that can survive salt content of irrigation water up to 5 g/L total dissolved solids. A true halophyte is considered to remain viable and complete its life cycle at seawater salinity (Rengasamy et al. 2003; Flowers and Colmer 2008).

Aronson (1989) compiled a list of halophytes with 1,560 species under 550 genera belonging to 117 families, which includes only food, forage, fuelwood, or soil stabilization crops. Chenopodiaceae family is the largest with over 275 as halophyte species. The other three superfamilies, Poaceae (grasses), Fabaceae (legumes), and Asteraceae (composites) contained 5 % of their species as halophytes. However, they represent less than 5 % of the species in these families (Aronson 1989). Among the monocot halophytes, Poaceae contains more halophytic genera (45 genera) than any other family, followed by Cyperaceae, which contains about 83 species. Flowers et al. (1977) showed probable relationships of halophytes from primitive (e.g., Laurales, Nymphales) to advanced (Asterales, Orchidales) orders on the basis of their order of flowering. Weiglin and Winter (1991) correlated leaf anatomy with zonation of 13 halophytes along a transect in a salt marsh in Jordan and based on the transectorial difference in photosynthetic pathway, degree of succulence etc. and classified them as euhalophytes (true halophytes), pseudohalophytes (salt avoiders), and crinohalophytes (salt excretors). Few species, i.e., *Salicornia bigelovii* can grow well and set seed even on soil solution exceeding 70 g/L total dissolved solids (TDS, i.e., 1.3 M NaCl) (Glenn et al. 1999). Some crop plants, i.e., sugar beet, date palm, and barley are also considered halophytes as they can be cultivated on irrigation water with 5 g/L TDS (Ayers and

Wescott 1989). On the other hand, most sensitive crops such as rice and bean can be severely affected by as low as 20–50 mM NaCl (Greenway and Munns 1980).

Halophytes have the abilities to tolerate high salt concentrations of the soil by maintaining more negative cellular water potential to absorb water from a soil solution of low water potential. This unique ability is achieved by a number of mechanisms that are found operational in halophytes. Due to their multiple origins, halophytes differ widely in their degree of tolerance as well as adaptability to salt (Ungar 1991). On the other hand, they share a common ability to accumulate large amounts of Na^+ in vacuoles as the major plant osmoticum (Khan et al. 2000; Moghaieb et al. 2004) simultaneously to inhibit K^+ absorption (Zhu 2003). This requires a functional Na^+/H^+ antiport system in the tonoplast and specially adapted membrane lipids to prevent leakage of Na^+ from the vacuole to the cytoplasm. Halophytes have relatively high rates of net Na^+ uptake ($1\text{--}10 \text{ nmol Na}^+ \text{ g}^{-1} \text{ fr. root s}^{-1}$) without injury to the plant. Na^+ influx was first measured in a halophyte, *Spergularia marina*, which showed uptake of $^{22}\text{Na}^+$ into whole plants in “steady state” salinity for over 2 h (Flowers and Colmer 2008). Halophytes have much lower Na^+ influx than the glycophyte *Arabidopsis* (i.e., $30 \text{ nmol g}^{-1} \text{ fr. root s}^{-1}$).

Oil-seed crops, such as *Kosteletzkya virginica* (Ruan et al. 2008); *Salvadora persica* (Reddy et al. 2008); *Salicornia bigelovii* (Glenn et al. 1991); and *Batis maritima* (Marcone 2003); fodder crops, such as *Atriplex* spp. (El-Shaer 2003) and *Distichlis palmeri* (Masters et al. 2007) are some of the agronomically useful halophytes. In spite of having high salt content from 15 to 50 % of leaf dry matter, halophytes generally contain high protein content (10–20 % of dry matter), and are considered as poor energy resource (Le Hou  rou 1996). Unlike leaves, the seeds of halophytes have a very low salt content, even under saline irrigation. The seeds of most halophytic species germinate usually during spring or during high precipitation, when soil salinity levels are reduced, but they germinate better under saline conditions. Elucidation of the mechanism of seed germination in halophytes under salinity will be extremely beneficial in developing salt-tolerant crops (Aslam et al. 2011). Salt-tolerant biofuel halophytes can also be grown on marginal agricultural land (Qadir et al. 2008) and could be irrigated with brackish water or seawater (Rozema and Flowers 2008). For revegetation and remediation of salt-affected regions to develop sustainable agricultural practice, we also need to rely on halophytes (Peacock et al. 2003). Previous studies also reported that some seagrasses and salt marsh plants have the ability to extract heavy metals from sediments (Cambrolle et al. 2008; Lewis and Devereux 2009). Salt-tolerant plants also improve water conductance, soil fertility (Qadir et al. 2008), and lower the water table (Barrett-Lennard 2002). The monocot halophyte *Puccinellia tenuiflora* can thrive in the saline-alkali soils and has an outstanding nutritional value for live-stocks (Yu et al. 2011). It accumulates inorganic ions, proline, betaine, and organic acid for osmotic adjustment (Guo et al. 2010). It is however imperative that the candidate species be assessed to determine the future plants crucial in evolving any economic crop halophyte. The processes and potential applications of halophytes merit much greater emphasis on their research and development for the improvement of salt tolerance traits of crop species.

9.3 Halophytes Versus Glycophytes

Plants have been classified as glycophytes or halophytes based on their ability to grow on high salt medium. Most of the plants cannot tolerate high salt concentrations of the soil and thus cannot be grown on these areas (Parvaiz and Satyawati 2008). These plants are called glycophytes, nonhalophytes, or non-salt-tolerating plants (Xiong and Zhu 2002). Only 2 % of terrestrial plant species have the ability to grow under salinity; such plants are known as salt-resistant plants, salt-tolerant plants, or halophytes (Flowers et al. 1986). The leaves of glycophytes are unable to retain high levels of salt without injury, whereas halophytes prefer to accumulate up to 50 % of shoot dry weight salt in their leaves in order to balance the osmotic potential of the salts outside the plant (Flowers et al. 1986). The optimal growth of a halophyte is observed at soil salinity ranging between 200 and 400 mM NaCl (Khan et al. 2005), where most crop plants would die rapidly. Due to lack of this adaptive mechanism, glycophytes are unable to survive in environments where halophytes thrive. However, there is no correlation detected between Na^+ accumulation and salinity tolerance in glycophytes (Shabala and Cuin 2007). Both halophytes and glycophytes are impacted in a similar fashion under high soil salinity, i.e., delay in the germination and reduction in the seed number (Ungar 1996). Also the fundamental plant metabolic processes, such as photosynthesis and respiration, are equally sensitive to salts (Volkmar et al. 1998). However, halophytes differ from the glycophytes in their ability to survive under a salt shock, i.e., tidal or rainfall events, which allows halophytes to develop a steady growth rate in a saline environment (Niu et al. 1993). Halophytes have a comparative advantage over glycophytes in their ability to determine the nature of transporters involved in the uptake of Na^+ (Wang et al. 2007). Such differences in the mode of Na^+ uptake by different species suggest evolutionary differences among species to thrive under same conditions.

Halophytes when grown in nonsaline (e.g., *S. maritima*) or less saline (i.e., 10 mM, e.g., *Halosarcia pergranulata*) culture solutions accumulate high concentrations of K^+ (Flowers and Colmer 2008). However, K^+ cannot substitute for Na^+ in all halophytes although it was reported to inhibit the growth of halophytes (Ramos et al. 2004; Flowers and Colmer 2008). Similar to the glycophytes, plant K^+ concentrations fall as Na^+ concentrations rise when halophytes are transferred from high K^+/Na^+ ratio (normal solution) medium to low K^+/Na^+ ratio (saline solution). To understand the salt tolerance mechanism and its evolution, it will be important to determine whether visible differences in net $S_{\text{K,Na}}$ between species reflect variations in their physiological and biochemical bases of salt tolerance. Still, very little is known about how Na^+ enters halophyte cells and tissues (Cheeseman 1988). However, in glycophytes, two mechanisms are thought to be responsible for Na^+ entry. In first type, (i.e., in rice), if the concentration of external solution is greater than 50 mM NaCl, Na^+ can be carried to the shoot via bypass flow through endodermis and enters the transpiration stream directly to concentrate the leaves. Secondly, (i.e., in wheat), Na^+ leaks into the plant symplastically via the root cortical cells by competitive binding to K^+ transporters or other cation channels (Rubio et al. 1995). This allows

entry of toxic levels of Na^+ into the plant, and depresses the uptake of K^+ . Salt-tolerant glycophytes activate pre-existing tonoplast antiporters under salt, whereas halophytes constantly activate vacuolar antiporters, even in the absence of NaCl (Glenn et al. 1999).

9.4 Monocot Versus Dicot Halophytes

Unlike monocotyledonous halophytes, the growth of dicotyledonous halophytes is found to be stimulated by salt. Several (but not all) dicotyledonous halophytes show optimal growth at salinity within 50–250 mM NaCl concentrations (Flowers et al. 1986), while monocotyledonous halophytes generally grow optimally either in the absence of salt or at salinity level of less than 50 mM NaCl concentration (Glenn et al. 1999). Generally, monocotyledonous halophytes show growth retardation at 170 mM salt concentrations. A few halophytes, such as *Plantago maritime* (Erdei and Kuiper 1979), *Atriplex* spp. (Longstreth and Nobel 1979), and *Lasthenia glabrata* (Kingsbury et al. 1976) do not show any increase in their growth at high salt concentrations (300 mM NaCl), but they are still able to complete their life cycles. Tall wheatgrass (*Agropyron elongatum*) is a halophyte relative of wheat, and is one of the most salt-tolerant monocotyledonous species, which grows optimally at salt concentration equivalent to seawater. Relative growth rates (dry mass) of dicot halophytes in 200–360 mM NaCl ranges between 6 and 160 $\text{mg g}^{-1} \text{d}^{-1}$, while that of monocot halophytes, it is between 15 and 26 $\text{mg g}^{-1} \text{d}^{-1}$ (Debez et al. 2006; Harrouni et al. 2003). Osmotic adjustment in dicot halophytes is achieved by means of ions accumulated by the root, which is associated with their succulence. But in monocot halophytes, which have lower water content, sugars may play a significant role. Estimates of the net $\text{K}^+:\text{Na}^+$ selectivity (net $S_{\text{K,Na}}$) indicates differences between monocot and dicot halophytes. Net $S_{\text{K,Na}}$ is calculated as the ratio of K^+ concentration in the plant to that in the medium divided by the ratio of Na^+ concentration in the plant to that in the medium, which ranges between 9 and 60 (Flowers and Colmer 2008). In only Poales the net $S_{\text{K,Na}}$ values are of the order of 60, whereas in Alismatales, the average net $S_{\text{K,Na}}$ is 16 (Flowers and Colmer 2008). The net $S_{\text{K,Na}}$ in monocot halophytes is twice and four times that of dicot halophytes at low and high salinity, respectively. However, many monocot halophytes have Na:K ratios of about one or less (Flowers et al. 1986). The monocots *Triglochin* and *Posidonia* are exceptions of having Na: K ratio and water content alike dicots, which may be the characteristic of Najadales within Monocotyledoneae (Tyerman et al. 1984).

Dicot halophytes are more succulent than the monocot halophytes because of their larger vacuolar volume. However, Juncaginaceae among the monocots are reported to be succulent. Earlier it was thought that normal dicot halophytes accumulate more NaCl in shoot tissues than monocot halophytes, and they were termed as “includers” and “excluders,” respectively (Ahmad et al. 1981). However, further studies on halophytes showed that monocot halophytes use Na^+ uptake into leaves,

similar to dicot halophytes (Rubio et al. 1995; Fricke et al. 1996). But, Na^+ uptake per unit of growth and requirement in monocot halophytes is less as compared to the dicot halophytes because of their lower cell vacuolar volume and leaf water content, so they maintain lower $\text{Na}^+:\text{K}^+$ ratio in saline conditions (Glenn et al. 1999). Yet, no detailed analysis of the effects of salinity on monocotyledonous halophytes has been reported. The differences of Na^+ and K^+ usage between mono- and dicotyledonous halophytes and the importance of cereals as crops, it would be pertinent that monocotyledonous halophytes (i.e., species of *Thinopyrum*, *Hordeum*, *Distichlis*, *Spartina*, and *Puccinellia*) be investigated further.

9.5 Physiology of Salinity Tolerance

Salt stress affects crop growth, development, and yield by imposing ion toxicity and imbalance, osmotic stress, nutritional disorders, membrane disorganization, metabolic toxicity, and inhibition of photosynthesis (Munns 2002; Zhu 2001). Two main consequences for plants growing in salinity are physiological drought, created by low osmotic potential of substrate compared to that inside the plant, and specific ion toxicities (Khan et al. 2006; Flowers and Colmer 2008). However, physiological basis of salt tolerance is present in all plants, but plant species show a great variation in salt tolerance ability; 25 mM NaCl can be toxic to certain glycophytes, whereas halophytes can tolerate salt stress level of 500–1,000 mM NaCl (Flowers et al. 2010). It is clear that tight regulation of ion transport from roots to shoots is vital for salt tolerance in halophytes (Munns 2005). The adaptation towards salt stress is an organized physiological adjustment at both cellular and molecular levels, which include ion homeostasis, osmotic adjustment, ion extrusion, and compartmentalization (Zhu 2001). The decline in plant growth at high-salinity level could be due to reduced carbon fixation, i.e., changes in biomass allocation between leaves, stem and root, which alters the balance of photosynthesis and respiration (Lovelock and Ball 2002). Another reason is fall in turgor due to high concentrations of ions in the apoplast (Balnokin et al. 2005; James et al. 2006), or change in cell wall elasticity (Touchette 2006). Other possibilities are related to osmotic adjustment—inability to either distribute or synthesize organic solutes, such as proteins, sugar, amino acids (Britto and Kronzucker 2006; Touchette 2007; Munns and Tester 2008). Accumulation of these compatible solutes is vital for cell osmo-regulation, protection of subcellular structures (Munns 2002), and maintenance of protein structures (Araújo et al. 2006).

Osmotic adjustment plays an important role for providing tolerance in halophytes under salinity (Flowers and Colmer 2008). Decreasing water potential (Ψ) must be established for water to flow through the soil-plant-atmosphere continuum. Thus, with increasing salinity plants must generate increasingly lower Ψ to allow continued water uptake (Touchette et al. 2009). This is achieved by increasing solute concentrations within the plant (Touchette 2007; Flowers and Colmer 2008).

Little, however, is known about how plant–water relations are altered in halophytes with rapid and substantial changes in soil salinity. Changes in tissue elasticity can also play an important role in plant–water relations during osmotic stress (Touchette 2007). The loss of water through transpiration can substantially decrease Ψ in ridged tissues, thus minimizing overall tissue water depletions (Touchette 2006). The general physiology of halophytes has been reviewed occasionally (Breckle 2002; Jithesh et al. 2006; Touchette 2007). The potential of halophytes as donors of tolerance for cereals and other crops has also been reviewed (Colmer et al. 2005, 2006). It is obvious that salt tolerance in halophytes is a complex mechanism and requires a combination of many different processes (Flowers and Colmer 2008). Understanding the physiology of halophyte plants is very important to help solve the problem of salinity in agricultural and horticultural crops.

9.5.1 *NaCl Uptake and Sequestration in Halophyte Cells*

Plant cells respond to external high salinity both by increasing sodium efflux at the plasma membrane and by the accumulation of sodium in the vacuole (Zhu 2000). Since monovalent ions are toxic at the concentrations required for osmotic adjustment, these (Na^+ and Cl^-) are compartmentalized, predominantly in vacuoles in the halophytes, so that their concentrations in the cytoplasm are maintained within tolerable limits (Subudhi and Baisakh 2011). Due to its low concentration in the cytoplasm, Na^+ is actively pumped into the vacuole from the cytoplasm, whereas Cl^- enter passively via anion channels to balance electrical charge differences across the membrane (Pantoja et al. 1992). However, the extent of ion accumulation and the degree of salt tolerance differ widely among halophytes (Glenn et al. 1996). Tonoplast antiporter activity has been reported in roots and leaves of *Atriplex nummularia* (Hassidim et al. 1990), *Plantago maritime* (Staal et al. 1991), *Atriplex gmelini* (Matoh et al. 1989), and *Mesembryanthemum crystallinum* (Barkla et al. 1995). The vacuolar Na^+/H^+ antiport appeared to be either constitutive or is found to be activated by high NaCl concentrations. A cytoplasmic Cl^- concentration was found to be from 25 to 150 mM in halophytes (Flowers et al. 1986). However, vacuoles of halophytes can accumulate 200–1,000 mM Cl^- with no expense of cellular energy.

9.5.2 *Retention of NaCl in Halophyte Vacuoles*

Na^+ is liable to leak back to the cytoplasm from the vacuole due to sharp concentration gradient between the two compartments (Maathuis et al. 1992). Cl^- would leak only when the vacuole remain no longer positively charged with respect to the cytoplasm. Isolated vacuoles of *Suaeda maritima* showed highly saturated fatty

acids and other lipids in tonoplast for minimizing permeability to NaCl (Leach et al. 1990). Further, tonoplast cation channels were also found to be closed at physiological concentrations of Na^+ ; only a small proportion of tonoplast H^+ ATPase activity maintains NaCl compartmentation (Maathuis et al. 1992). Hence, halophytes do not spend much of metabolic energy, and thus have the potential of maintaining high yield during high salinity. In *Aneurolepidium chinense*, a monocot halophyte, membrane protein (*AcPMP3*), localized in the root cap, which acts as a regulator of accumulation of Na^+ and K^+ (Inada et al. 2005).

9.5.3 Salt Inclusion Versus Exclusion

Many halophytes use salt as an osmoticum to balance external medium concentration (Ungar 1991). In halophytes, salt exclusion is the most common mean of surviving under high salt concentrations (Waisel et al. 1986). Halophytes excrete more than 50 % of the salt entering the leaf (Warwick and Halloran 1992). Mangrove plants exclude 99 % of the salts through the roots (Tomlinson 1986), and the casparian strips play a role in salt exclusion from the inner tissues (Flowers et al. 1986). However, there is no obvious correlation between salt exclusion and salt tolerance. In glycophytes, the slow growth rate of the leaves is due to their inability to utilize the salt transported from the root. Majority of crop plants, many dicotyledonous halophytes and most of the monocotyledonous halophytes are excluders, and only a few dicotyledonous halophytes are includers. It is surprising that the domesticated plants selected for salt tolerance by breeders are monocot excluders, while naturally salt-tolerant species are mostly includers (O'Leary 1995).

9.5.4 Na^+/K^+ Discrimination

Due to their competitive ionic interactions, Na^+ and Cl^- can suppress net nutrient uptake or affect membrane integrity. High levels of Na^+ lead to K^+ deficiencies (Tester and Davenport 2003). The ionic stress in plants under salinity is usually due to high Na^+/K^+ and $\text{Na}^+/\text{Ca}^{2+}$ ratios, and accumulation of toxic Na^+ and Cl^- in tissues, which impairs cellular metabolism (Blumwald et al. 2000; Zhu 2003). Regulation of ion selectivity, especially Na^+/K^+ discrimination is associated with salt tolerance (Volkmar et al. 1998). Because similar uptake mechanisms operate for both Na^+ and K^+ , the former can be substituted for the latter (Schroeder et al. 1994). A high level of K^+ in young tissues is associated with salt tolerance in several species (Khatun and Flowers 1995). Generally, monocot halophytes accumulate high levels of K^+ than Na^+ in their leaves (Albert and Kinzel 1973; Albert and Popp 1977). A positive relationship is believed to exist between Na^+ inclusion and salt tolerance in includer halophytes (Clipson and Flowers 1987). In such plants,

K⁺ accumulation was observed to be almost 4 % of total cations, and their Na⁺/K⁺ ratios were as high as 30 at seawater level salinity (Naidoo and Rughunanan 1990). Some nonhalophyte species discriminate against Na⁺ more than others by lowering the Na⁺ concentrations in their leaf cytoplasm (Hajibagheri et al. 1989). The gene controlling Na⁺/K⁺ discrimination was identified in *Triticum* that confers increased discrimination and enhanced salt tolerance when introduced through recombination in other species (Dvorak et al. 1994). Na⁺/K⁺ discrimination, however, is not a requirement for salt tolerance in glycophytes, such as barley and wheat (Volkmar et al. 1998).

9.5.5 Plasma Membrane Antiporters and ATPases

Both halophytes and glycophytes have the ability to export Na⁺ from the cytoplasm to the extracellular space using plasmamembrane Na⁺/H⁺-antiporters, while H⁺-ATPases provide the H⁺ electrochemical gradient at the plasmalemma (Niu et al. 1995). *Atriplex nummularia* and *Salicornia bigelovii* showed higher activity of root plasmalemma antiporter in plants grown on 400 mM NaCl as compared to control (Hassidim et al. 1990; Lin et al. 1997). NaCl was also found to induce a P-type H⁺-ATPase that supply energy for the antiporter (Niu et al. 1995). This also clarifies how halophytes expel Na⁺ from the cell. However, the PM-ATPase activity does not primarily determines salt tolerance, and long-term tolerance is due to an increase in the resistance of the root cells to Na⁺ entry. Transport of ions across the tonoplast is energized by a proton motive force (PMF) generated by the vacuolar H⁺-ATPase (V-ATPase) and H⁺-pyrophosphatase (V-PPase) (Gaxiola et al. 2007). Na⁺/H⁺ exchange in vesicles was found to be constitutively high and increased with salinization of the plant (Barkla et al. 2002). In *Salicornia bigelovii*, the activity of PM- and V-ATPases along with V-PPase increased upon addition of NaCl to the growth medium (Parks et al. 2002). However, in *Suaeda salsa*, salinity increased the activity of the V-ATPase and Na⁺/H⁺ antiporter in tonoplast vesicles (Qiu et al. 2007), rather than the V-PPase (Wang et al. 2001). In *Thellungiella halophila*, NaCl increased the Na⁺/H⁺ antiporter in vesicles but not in plasma membrane (Vera-Estrella et al. 2005).

9.5.6 Accumulation of Osmolytes

Accumulation of metabolically compatible solutes (organic compounds) in the cytoplasm is an important role in salt tolerance mechanism at the cellular level, which balances the osmotic potential of the Na⁺ and Cl⁻ accumulated in the vacuole (Wyn-Jones and Gorham 2002). Halophytes synthesize a range of compatible solutes from quaternary ammonium compounds, methylated sulphonio compounds (sugars, such as sucrose), methylated proline-related compounds

(methyl-proline), betaines (glycinebetaine), and amino acids (proline) to sugar alcohols (sorbitol) depending on phylogeny and functional needs (Hasegawa et al. 2000; Rhodes et al. 2002; Flowers and Colmer 2008). The polyhydric alcohols (polyols) exist in both acyclic and cyclic forms and are believed to be associated with plant salt tolerance (Bohnert and Shen 1999). Commonly found polyols in plants are acyclic forms, such as mannitol, glycerol and sorbitol, and cyclic (cyclitols) forms, such as ononitol and pinitol. Polyols make up a substantial percentage of all assimilated CO₂ by acting as scavengers of stress-induced free oxygen radicals (Bohnert et al. 1995).

A few halophytes accumulate specific compounds: *Melanleuca bracteata* accumulates proline analogue 4-hydroxy-*N*-methyl proline (MHP) and *Spartina* spp. accumulate dimethylsulphoniopropionate (DMS) (Naidu et al. 2000). Such proline analogues increase plant survival under salinity because of their ability to cause regulation, compartmentalization, and production outlay (Bohnert and Shen 1998). However, there is no clear pattern of a particular group of solutes being exclusive to a particular order in other species (Naidu 2003). The concentrations of organic solutes are known to increase several fold (20–80 times) with increasing external NaCl (Ishitani et al. 1996). In a few cases, there is no change in the concentrations of compatible solutes with increasing external salinity, suggesting their constitutive synthesis within the species (Khan et al. 1998). Some organic solutes might redistribute between compartments with increase in salinity, with no significant effect on a whole-tissue basis. However, if confined to the cytoplasm, these solutes can contribute significantly within that volume (Rhodes et al. 2002). This avoids ions toxicity, but the process requires more energy compared to osmotic adjustment with inorganic ions (Ashraf and Harris 2004). Another way of osmotic adjustment in halophyte is by aquaporin water channel, which is involved in intracellular compartmentalization of the water. These aquaporins were found to play important roles in salt tolerance by maintaining osmotic homeostasis and plant cell turgor under salt stress (Maurel 1997).

9.5.7 Other Mechanisms of Salt Tolerance

Secondary mechanisms in halophytes to handle salt stress involve salt glands (Weber 2008), salt bladders (Freitas and Breckle 1992) and succulent tissues (Yeo and Flowers 1986). Salt excretion by salt glands of halophytes is reported to be the fastest ion transport systems in plants (Pollak and Waisel 1979). Salt glands have been described in a few orders, such as Poales (*Aeluropus littoralis* and *Chloris gayana*), Myrtales (*Laguncularia racemosa*), Caryophyllales (*Mesembryanthemum crystallinum* and *Atriplex halimus*), Lamiales (*Avicennia marina* and *Avicennia germinans*), and Solanales (*Cressa cretica*). Salt glands are found on the epidermis of every aerial part but are more concentrated in leaves, and these glands are rich in mitochondria and other organelles but lack a central vacuole (Waisel 1972). The water evaporates

through these salt glands and the salt remains on the leaf surface in crystalline forms, which are blown away through wind or by rain (Lipscshitz et al. 1974). It is a common way of salt avoidance (Waisel et al. 1986). Plants grown under high salinity tend to shed the old leaves as another strategy to avoid the toxic effects of excess sodium salts (Aslam et al. 2011). Ion recirculation from shoots to the roots is also an important salt tolerance mechanism as the toxic ions would have to be carried in the symplasm of the phloem (Munns and Tester 2008).

Succulence dilutes excess NaCl in the leaf tissues (Kramer 1984), but reduction in leaf water content concentrates NaCl in the cell sap, thus supporting osmotic adjustment when halophytes are grown under high salinity (Glenn 1987). Succulence causes increase in cell size, decrease in growth extension, decrease in surface area per tissue volume and higher water content per tissue volume (Weber 2008). The leaves of succulent plants have more and larger mitochondria indicating some extra energy consumption for salt compartmentalization and excretion (Siew and Klein 1969). Succulence is extremely rare in monocots, and only 15 % of monocotyledonous halophytes have salt glands. Halophytes also increase their water use efficiency in response to salt, thereby minimizing the amount of transpired water (Glenn et al. 1997). In *Aster tripolium*, a unique feedback control mechanism of stomatal opening by apoplastic Na^+ in the leaves was identified to regulate maintenance of leaf water potential (Perera et al. 1997).

Seed germination and early seedling vigor, the determinant of population survival and progeny maintenance, are very sensitive to minor environmental disturbance, which in most cases can be lethal (Khan and Gul 2006). The seeds of halophytes have developed strategies to remain dormant until the favorable conditions for germination and early seedling stage are available (Khan and Gul 2006). *Salicornia utahensis* shows the behavior of a true halophyte, where seedling growth at 1,000 mM NaCl was equal to that in control and growth stimulation was observed up to 600 mM NaCl (Gul et al. 2009).

9.6 Breeding Approaches Towards Salt Tolerance

It is not clear how salinity reduces the growth of plants. The accumulation of Na^+ and Cl^- in crop plants reduces their growth and enhances senescence of mature leaves, resulting in a reduction in functional leaf area and yield (Munns and Tester 2008). Therefore, increasing salt tolerance of crop plants is needed for sustaining yield in salt-affected areas. However, complexities of salt tolerance mechanisms always hinder breeding of salt-tolerant crops. So, despite huge efforts ranging from genetic crossing to transgenics towards increasing salt tolerance in crops, only few productive salt-tolerant varieties have so far been developed (Witcombe et al. 2008). Genomic studies on halophytes may help identify specific salt tolerance traits that may be selectively targeted for improvement in crop plants (Huang et al. 2008), which in the long run may lead to the domestication of salt-tolerant genes in economic crop plants. Halophytes respond to salt stress at cellular, tissue, and whole

plant level. Thus, a complete understanding of plant salt tolerance will require studying the mechanisms involved at each level.

The taxonomic diversity of halophytes offers a rich source of germplasm, which suggests a possibility that through wide crosses salt tolerance can be introduced into crop plants (Epstein et al. 1980). Boyko and Boyko (1959) first demonstrated that salt tolerance in crop plants could be increased by crossing them with their salt-tolerant relatives (e.g., wheat \times *Agropyrum*). In addition, a universal trait of halophyte may represent its convergent evolution for salt tolerance, and such a trait can be a candidate for transfer from halophytes to glycophytes (Zhu et al. 1997). Few halophytes have desirable crop characteristics that can be introgressed into crop plants through conventional breeding to make them useful for agriculture. Few economically important crops with salt-tolerant, wild relatives are wheat (*Aegilops* and *Thinopyrum*; Gorham and Wyn-Jones 1993), barley (*Hordeum maritimum*; Aronson 1989), tomato (*Lycopersicum cheesmanii* and *L. pimpinellifolium*; Asins et al. 1993), and *Beta vulgaris* ssp. *vulgaris* (*B. vulgaris* ssp. *Maritime*; Rozema et al. 1993). In wheat, few reports are available of transferring salt tolerance between species (Glenn et al. 1999). Transferring *Kna1* gene (for enhanced K^+/Na^+ discrimination) from *Triticum aestivum* to *T. turginsum* through conventional crossing slightly improved salt tolerance of wheat (Dvorak et al. 1994). Wide crosses of wheat with their halophytic relatives provide an alternative approach to raise amphiploids with increased glycinebetaine in the leaves (Islam et al. 2007). However, due to multigenic nature of halophyte salt tolerance, glycophyte-halophyte crosses are yet to be fulfilled (Flowers and Yeo 1995).

Conventional breeding techniques with agronomical parameters related to growth and yield have been used to improve salinity tolerance in crop plants (Ashraf 2002; Ashraf and Harris 2004). Thus far, SKC1 is the only gene that has been used in marker-assisted breeding of salt tolerant rice, although ongoing fine mapping of the salt tolerance QTL “saltol” will lead identification of more markers to facilitate development of salt-tolerant rice cultivars (saltol, Thomson et al. 2010). But, salt tolerance is a multigenic trait in both halophytes and glycophytes (Baisakh et al. 2012). Therefore, conventional breeding for yield has not brought many salt-resistant varieties of the field crops (Witcombe et al. 2008). The probability of combining traits to maximize yield under saline conditions is very low. Thus, it was advocated to understand the physiology of salt tolerance and assemblage of a variety of traits for better results (James et al. 2008). Due to the complexity of the mechanism, transfer of one or two genes has generally not been successful to enhance the tolerance of transgenic plants. However, there are instances where single gene transfers appear to have altered yield (Baisakh et al. 2012; Table 9.1). Study of halophytes can be useful for breeding salt tolerance because: (1) mechanisms of survival and productivity maintenance on saline water can define the required adaptations in tolerant germplasm (Zhu et al. 1997); (2) growing halophytes in an agronomic pattern can be used to find a source of tolerant germplasm for high-salinity agriculture; and (3) halophytes can be used as a direct source of new crops (Glenn et al. 1997).

Table 9.1 Translation of genes from halophyte plants in model and/or crop plants for improvement of salt tolerance

Gene	Halophyte source	Recipient plant	References
<i>Ion transporters</i>			
Na ⁺ /H ⁺ antiporter	<i>Thellungiella halophila</i>	<i>Arabidopsis</i>	Wu et al. (2009)
Na ⁺ /H ⁺ antiporter	<i>Chenopodium glaucum</i>	Rice	Li et al. (2008)
Na ⁺ /H ⁺ antiporter	<i>Arriplex gmelini</i>	Rice	Ohta et al. (2002)
Na ⁺ /H ⁺ antiporter and H ⁺ -PPase	<i>Suaeda salsa</i>	Rice	Zhao et al. (2006)
Na ⁺ /H ⁺ antiporter (<i>NHX1</i>)	<i>Salicornia brachiata</i>	Tobacco	Jha et al. (2011)
Vacuolar Na ⁺ /H ⁺ antiporter (<i>NHX1</i>)	<i>Hordeum brevisulatum</i>	Tobacco	Lu et al. (2005)
Vacuolar Na ⁺ /H ⁺ antiporter (<i>NHX1</i>)	<i>Halostachys caspica</i>	<i>Arabidopsis</i>	Guan et al. (2011)
Vacuolar Na ⁺ /H ⁺ antiporter (<i>NHX1</i>)	<i>Salsola soda</i>	Alfalfa	Li et al. (2011)
Vacuolar Na ⁺ /H ⁺ antiporter (<i>NHX1</i>)	<i>Agropyron elongatum</i>	<i>Arabidopsis</i>	Qiao et al. (2007)
Vacuolar Na ⁺ /H ⁺ antiporter (<i>NHX1</i>)	<i>Aeluropus littoralis</i>	Tobacco	Zhang et al. (2008c)
Vacuolar Na ⁺ /H ⁺ antiporter (<i>NHX1</i>)	<i>Leptochloa fusca</i>	Tobacco	Rauf et al. (2014)
Vacuolar Na ⁺ /H ⁺ antiporter (<i>NHX2</i>)	<i>Suaeda salsa</i>	<i>Arabidopsis</i>	Li et al. (2009, 2012a)
Vacuolar Na ⁺ /H ⁺ antiporter (<i>NHX2</i>)	<i>Hordeum vulgare</i>	<i>Arabidopsis</i>	Bayat et al. (2011)
Vacuolar-H ⁺ -pyrophosphatase	<i>Suaeda salsa</i>	<i>Arabidopsis</i>	Guo et al. (2006)
Vacuolar-H ⁺ -pyrophosphatase	<i>Suaeda corniculata</i>	<i>Arabidopsis</i>	Liu et al. (2011)
Vacuolar-H ⁺ -pyrophosphatase	<i>Halostachys caspica</i>	<i>Arabidopsis</i>	Hu et al. (2012)
Vacuolar-H ⁺ -pyrophosphatase	<i>Kalidium foliatum</i>	<i>Arabidopsis</i>	Yao et al. (2012)
H ⁺ -PPase	<i>Thellungiella halophila</i>	Tobacco	Gao et al. (2006)
H ⁺ -PPase	<i>Thellungiella halophila</i>	Cotton	Lv et al. (2008)
Tonoplast pyrophosphatases gene (<i>VP1</i>)	<i>Chenopodium glaucum</i>	<i>Arabidopsis</i>	Hu et al. (2009)
AKT-1-type-K ⁺ channel	<i>Puccinellia tenuiflora</i>	<i>Arabidopsis</i>	Ardie et al. (2010)
Vacuolar H ⁺ -ATPase subunit c1 (<i>SaVHAc1</i>)	<i>Spartina alterniflora</i>	Rice	Baisakh et al. (2012)
Vacuolar H ⁺ /Ca ²⁺ Transporter	<i>Suaeda salsa</i>	<i>Arabidopsis</i>	Han et al. (2012)
Tonoplast AQP gene (<i>TsTIP1.2</i>)	<i>Thellungiella salsuginea</i>	<i>Arabidopsis</i>	Wang et al. (2014)
ADP ribosylation factor 1 (<i>SaARF1</i>)	<i>Spartina alterniflora</i>	<i>Arabidopsis</i> , rice	Joshi et al. (2014)

<i>Osmolyte biosynthesis genes</i>			
myo-Inositol O-methyl transferase	<i>Mesembryanthemum crystallinum</i>	Tobacco	Sheveleva et al. (1997)
myo-Inositol phosphate synthase (MIPS)	<i>Porteresia coarctata</i>	Brassica, rice	Das-Chatterjee et al. (2006)
myo-Inositol phosphate synthase (MIPS)	<i>Spartina alterniflora</i>	Tobacco, rice	Baisakh et al. (2009)
myo-Inositol phosphate synthase (SaINO1)	<i>Spartina alterniflora</i>	<i>Arabidopsis</i>	Joshi et al. (2013)
Proline transporter	<i>Arriplex hortensis</i>	<i>Arabidopsis</i>	Shen et al. (2002)
Inositol polyphosphate kinase	<i>Thellungiella halophila</i>	Brassica napus	Zhu et al. (2009)
Choline oxygenase	<i>Suaeda liaotungensis</i>	Tobacco	Li et al. (2003a)
Betaine aldehyde dehydrogenase (BADH)	<i>Suaeda liaotungensis</i>	Tobacco	Li et al. (2003b)
Betaine aldehyde dehydrogenase (BADH)	<i>Arriplex hortensis</i>	Tomato	Jia et al. (2002)
Betaine aldehyde dehydrogenase (BADH)	<i>Suaeda liaotungensis</i>	Maize	Wu et al. (2008)
<i>Antioxidative enzymes</i>			
Glutathione S-transferase	<i>Suaeda salsa</i>	Rice	Zhao and Zhang (2006a, b)
Glutathione S-transferase	<i>Limonium bicolor</i>	Yeast	Diao et al. (2010)
Metallothioneine and UDP-galactose epimerase	<i>Paspalum vaginatum</i>	Rice	Endo et al. (2005)
Chloroplastic ascorbate peroxidase (CHLAPX)	<i>Suaeda salsa</i>	<i>Arabidopsis</i>	Pang et al. (2011)
Peroxisomal ascorbate peroxidase (pAPX)	<i>Salicornia brachiata</i>	Tobacco	Tiwari et al. (2014), Singh et al. (2014)
Chloroplast-located Peroxiredoxin Q	<i>Suaeda salsa</i>	<i>Arabidopsis</i>	Jing et al. (2006)
Phytoene synthase	<i>Salicornia europaea</i>	<i>Arabidopsis</i>	Han et al. (2008)
Choline monooxygenase	<i>Salicornia europaea</i>	Tobacco	Wu et al. (2010)
β-Lycopene Cyclase (LCY)	<i>Salicornia europaea</i>	<i>Arabidopsis</i> , Tobacco	Chen et al. (2011)
Chloroplastic monodehydroascorbate reductase	<i>Avicennia marina</i>	Tobacco	Kavitha et al. 2010a, b
Stroma ascorbate peroxidase	<i>Suaeda salsa</i>	<i>Arabidopsis</i>	Li et al. (2012b)
Cytosolic Copper/zinc superoxide dismutase	<i>Avicennia marina</i>	Rice	Prashanth et al. (2008)

(continued)

Table 9.1 (continued)

Gene	Halophyte source	Recipient plant	References
<i>Signaling/regulatory pathways</i>			
Serine-rich protein	<i>Porteresia coarctata</i>	Finger millet	Mahalakshmi et al. (2006)
Allene oxide cyclase	<i>Bruguiera sexangula</i>	Tobacco	Yamada et al. (2002)
Cyclophilin (<i>CYP1</i>)	<i>Thellungiella halophila</i>	Tobacco	Chen et al. (2007)
CBL-interacting protein kinase (<i>CIPK2</i>)	<i>Hordeum brevisulatum</i>	<i>Arabidopsis</i>	Li et al. (2009)
Abscicic acid stress ripening-1 (<i>ASR-1</i>)	<i>Salicornia brachiata</i>	Tobacco	Jha et al. (2012)
Acetylcholinesterase	<i>Salicornia europaea</i>	Tobacco	Yamamoto et al. (2009)
Ankyrin repeat protein 1 (<i>ARP1</i>)	<i>Bruguiera gymnorhiza</i>	<i>Arabidopsis</i>	Miyama and Tada (2011)
Cacineurin B-like protein	<i>Thellungiella halophila</i>	<i>Arabidopsis</i>	Sun et al. (2008)
<i>Transcription factors</i>			
DREB1 (<i>EREBP/AP2</i> -type protein)	<i>Arriplex hortensis</i>	Tobacco	Shen et al. (2003)
<i>DREB1</i> (<i>DREB3a</i>)	<i>Leymus chinensis</i>	<i>Arabidopsis</i>	Xianjun et al. (2011)
Zinc finger protein 1	<i>Thellungiella halophila</i>	<i>Arabidopsis</i>	Xu et al. (2007)
A20/AN1 zinc-finger (Stress Associated Protein)	<i>Aeluropus litoralis</i>	Tobacco	Saad et al. (2010)
Zinc-finger-like (<i>ZFL</i>)	<i>Tamarix hispida</i>	Tobacco	An et al. (2011)
Late embryogenesis abundant (<i>LEA</i>)	<i>Tamarix hispida</i>	Tobacco	Qu et al. (2012)
Late embryogenesis abundant (<i>LEA</i>)	<i>Thellungiella salsuginea</i>	<i>Arabidopsis</i>	Zhang et al. (2012)
Basic leucine zipper (<i>bZIP</i>)	<i>Tamarix hispida</i>	Tobacco	Qu et al. (2012)
<i>MYB-1</i>	<i>Leymus chinensis</i>	<i>Arabidopsis</i>	Cheng et al. (2013)
<i>bHLH</i>	<i>Oryza rufipogon</i>	<i>Arabidopsis</i>	Li et al. (2010)
<i>Others</i>			
Uncharacterized universal stress protein gene	<i>Salicornia brachiata</i>	<i>E. coli</i>	Udawat et al. (2014)
Pathogenesis-related gene (<i>NPR1</i>)	<i>Malus hupehensis</i>	Tobacco	Zhang et al. (2014)
Unknown function gene (Bg70 and cyc02 homolog)	<i>Bruguiera gymnorhiza</i>	<i>Arabidopsis</i>	Ezawa and Tada (2009)

9.7 Understanding Molecular Mechanisms of Salt Tolerance in Halophytes

Conventional breeding techniques were reported to be less successful in transferring salt tolerance (Sairam and Tyagi 2004), largely due to the complexity of the physiological mechanisms, strong genotype by environment interaction for yield traits and lack of an efficient marker-assisted selection system (Flowers 2004). Therefore, an integrated strategy involving multiple genes engineering would be required for development and release of salt-tolerant crops (Bohnert et al. 2006).

In recent years, research on molecular mechanisms operating against different types of abiotic stresses is in progress (Aslam et al. 2011). Halophytes show a diversity of growth responses with increasing salinity and the mechanisms behind these remain to be resolved. Although genes/proteins involved in transport of Na^+ , Cl^- , and K^+ ions have been identified, how halophytes regulate the constancy of their ion concentrations with the coordinated network of gene regulation requires further investigation. Conventional breeding to improve salt tolerance has been less successful; it is therefore necessary to think on transferring halophyte genes into glycophytes directly. Recent strategy is to genetically modify plants with genes from halophytes to make them more salt tolerant (Aslam et al. 2011). The transgenic salt-tolerant plants were also found to have resistance against multiple abiotic stresses, such as chilling, freezing, heat, and drought (Zhu 2001). Therefore, identification of genes regulated under salt stress in halophytes will have major applications in engineering salt tolerance in crop plants. Although halophytes can adjust to sudden changes in external salinity, but how this change in initial response is sensed is still uncertain (Chinnusamy et al. 2005). Expressed sequence tags (ESTs) have been reported to be an efficient, rapid, and cost-effective strategy to discover novel genes involved in abiotic stress tolerance (Mehta et al. 2005; Baisakh et al. 2008), which helps in understanding the genetic mechanisms that control plant responses to a given ecological condition (Baisakh et al. 2006). Differential expression of transcript-derived fragments under salt stress through complementary DNA-AFLP showed salt stress-induction of genes that may have possible roles in salt tolerance of a monocotyledonous halophyte *Spartina alterniflora* (Baisakh et al. 2006). Comparative transcriptome analysis of *S. alterniflora* showed it to be closer to cereals like maize and rice than other halophytes at both DNA and protein levels.

Halophytes might use the transporters and regulatory networks same as in certain glycophytes, but with different set points. Therefore, comparative understanding of salt tolerance mechanisms in wide/wild relatives of crop plants will be vital for devising strategies to breed salt tolerance. Genetic manipulation of the promoter regions might induce the metabolic pathways related to compatible osmotica, which are blocked in glycophytes (Bohnert et al. 1995).

Undoubtedly, several genes are required for salt tolerance, but it is still interesting to understand the effect of single gene additions or alterations on glycophytes transferred from halophytes (Bohnert and Jensen 1996). Yet, very little is known about the signaling cascades regulating the synthesis of compatible solutes in plants (Chinnusamy

et al. 2005). The molecular basis of salinity-induced accumulation of some organic solutes has already been reported in few halophytes, e.g., inositol in *M. crystallinum* (Vernon et al. 1993), proline in *T. halophila* (Kant et al. 2006), and glycinebetaine in several halophytes. The expression of genes encoding for the first enzyme in the biosynthetic pathway of proline, the α 1-pyrroline-5-carboxylate synthetase (P5CS), increased in shoots and roots with increasing salinity, while the gene encoding the first step of the breakdown of proline, proline dehydrogenase (PDH), was found to be decreased (Kant et al. 2006). Overexpression of P5CS in transgenic tobacco plants showed resistance to drought, freezing, and salinity stresses (Hong et al. 2000; Nanjo et al. 2003). Glycinebetaine is another osmolyte, which is synthesized from choline in two steps by enzymes choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). Both BADH and CMO expression levels were enhanced during salt stress in *Atriplex nummularia*, *Suaeda aegyptiaca*, *Salicornia europaea*, and *S. maritima* (Moghaieb et al. 2004; Tabuchi et al. 2005) resulting in enhanced expressions of proteins related to glycinebetaine synthesis (Askari et al. 2006). However, in transgenics aimed at increasing glycinebetaine synthesis in plants, such as tobacco and rice that do not naturally contain this solute, precursor (choline) availability hinders glycinebetaine synthesis (Su et al. 2006).

Plants protect their cells from the damage by reactive oxygen species by induction of activities of antioxidative enzymes such as catalase, peroxidase, glutathione reductase, and superoxide dismutase (Mittova et al. 2003). Overexpression of genes, such as mitochondrial Mn-SOD, Fe-SOD, chloroplastic Cu/Zn-SOD, catalase, and glutathione-S-transferase (GST)/glutathione peroxidase (GPX) were found to increase the performance of plants under stress (Roxas et al. 2000).

A range of signal transduction pathways including SOS1 and Ca^{2+} has been identified in glycophyte models (Bartels and Sunkar 2005) and these should be further explored in halophytes (Flowers and Colmer 2008). Na^+ efflux from the cytoplasm is mediated by Na^+/H^+ exchange by SOS1 at the plasma membrane and/or NHX1 at the tonoplast level (Apse and Blumwald 2007). SOS1 acts as a sensor for cellular Na^+ and its overexpression through cytosolic Ca^{2+} upregulates ABA concentrations, which leads to an increase in reactive oxygen species (Chinnusamy et al. 2005). SOS1 is present in membrane vesicles of *Thellungiella halophila* shoots and roots and is highly expressed even in the absence of salt (Vera-Estrella et al. 2005). The similarity between the genomes of *T. halophila* and *Arabidopsis* has allowed identification of salinity-induced genes through comparative transcriptome profiling (Wong et al. 2006). The accumulation of Na^+ into vacuoles is mediated by Na^+/H^+ exchanger (NHX; Apse and Blumwald 2007), which was detected in the plasma membrane fraction of *T. halophila* roots (Vera-Estrella et al. 2005). Genes for the Na^+/H^+ antiporter were cloned from *S. salsa* (Ma et al. 2006) and *A. gmelini* (Hamada et al. 2001), and identified in *M. crystallinum* (Chauhan et al. 2000). Some ion-responsive antiporter/channel genes have been reported in monocot halophyte *Puccinellia tenuiflora*; *PutPMP3-1*, and *PutPMP3-2* encoding plasma membrane protein 3 family prevent the accumulation of excess Na^+ and K^+ ions (Zhang et al. 2008a). Similarly, *PutHKT2-1* encoding a high-affinity K^+ transporter facilitates K^+ uptake to maintain a high ratio of K^+/Na^+ in the cells (Ardie et al. 2009). *PutAKT1*,

a plasma membrane-localized K^+ channel interacts with KPutB1 to alter K^+ and Na^+ homeostasis (Ardie et al. 2011). PutCAX1, a tonoplast Ca^{2+}/H^+ antiporter mediates Ca^{2+} , Ba^{2+} , and Zn^{2+} transport (Liu et al. 2009) and PtNHA1 encoding Na^+/H^+ antiporter functions in the maintenance of low cytosolic Na^+ (Wang et al. 2011). K^+ is an essential element in protein synthesis as it binds tRNA to the ribosomes (Blaha et al. 2000). Homologs of K^+ transporters, such as AKT whose expression changed on salinity stress were identified in the halophyte *M. crystallinum* (Su et al. 2003).

Free polyamines concentrations, which are thought to regulate K^+ fluxes (Shabala et al. 2007), also increase salinity stress tolerance (Shevyakova et al. 2006; Kuznetsov et al. 2007). Osmotic stress induces several proteins in vegetative tissues, such as accumulation of late-embryogenesis-abundant (LEA) proteins (Parvaiz and Satyawati 2008), which indicates their protective role under dehydration stress (Liu et al. 1998). Only a few proteomic investigations have been reported in monocot halophytes (Sobhanian et al. 2010; Sengupta and Majumder 2009). Sobhanian et al. (2010) reported that *Aeluropus lagopoides*, a C_4 monocot halophyte, exhibited enhanced energy production, amino acid biosynthesis, C_4 photosynthetic pathway, and detoxification under saline conditions. However, the wild halophytic rice, *Porteresia coarctata*, shows enhanced energy and osmolytes production, alteration of transcriptional activity and protein modification, as well as change in cell wall components under salinity (Sengupta and Majumder 2009).

A number of transcription factors such as DREBs have been isolated from several plants (Agarwal et al. 2006), but only a few reports are available from halophytic plants like *Physcomitrella patens* (Liu et al. 2007), *Atriplex hortensis* (Shen et al. 2003) and *Salicornia brachiata* (Gupta et al. 2010). Certain genes being highly expressed under salinity but not under normal conditions suggests that their products may act as chaperons in protecting the cellular structure during stress (Xiong and Zhu 2002).

A number of genes isolated from halophytes have been transferred to glycophyte plants through different genetic transformation techniques. Transgenic plants with halophyte genes have shown positive results with their tolerance to salt and other abiotic stresses (Joshi et al. 2013, 2014). A comprehensive list of reports describing development of transgenic plants using halophyte genes is presented in Table 9.1 and hence has not been discussed in detail.

9.8 Stress-Inducible Promoters in Halophytes

A promoter determines the chronological and spatial order of gene expression. The choice of promoters significantly affects the expression of the genes in transgenic manipulation (Munns and Tester 2008). It was shown earlier that high constitutive expression of the foreign gene may be detrimental to the host plant, with increased sterility, retarded development, abnormal morphology, yield penalty, altered grain composition, and transgene silencing (Xu et al. 2006; Kanneganti and Gupta 2008). Strong, tissue-specific, or stress-inducible promoter to direct gene expression to a

required tissue, at a particular development stage and/or in response to a stress may circumvent this problem (Pino et al. 2007). Therefore, identification of stress-inducible promoters must be explored in halophytes for successful exploitation of genes for stress tolerance, (Aslam et al. 2011). It is necessary to use transformation vectors containing specific promoters to ensure the cloned gene expression in individual host cells. Further, it is essential to isolate and analyze the composition and function of the promoter for gene expression, regulation, and vector construction during genetic engineering (Li et al. 2007). At present, promoter analysis is a hotspot in genetic engineering and many promoters have been cloned and some of them were reportedly salt inducible.

Schaeffer et al. (1995) analyzed the sequences of the enhancer and the silencer in the regions of the *CAM* promoter in halophyte *Mesembryanthemum crystallinum*. Yin et al. (2002) obtained a salt-responsive *BADH* promoter from the halophyte *Atriplex centralasiatica* and found basic elements and some motifs, such as TATA-box, CAAT-box, GC-motif, TTCGACA (EIRE), TTATTACAA (MRE), TCCAAG, ACATTACGG, TCATTTCOA (WUN-motif), AGAAAAGTG (HSE), and ACCACGTAAG (ABRE), related to stress. Zhang (2002) isolated *Ped*, a promoter fragment, 243 bp in length with hyperactivity, from *Dunaliella salina* and concluded that this promoter fragment contains a conservative G-box, CAAT-box, and TAAT-box. Isolation and characterization of the promoter of *CMO* gene from *S. liaotungensis* was done to study the relationship between structure and function of this promoter and investigate the molecular mechanism of gene regulation (Li et al. 2007). Kavitha et al. (2010a, b) found the regulatory elements involved in drought, light, cold/freeze, and gibberellic acid response in the promoter of *AmMDAR* gene isolated from *Avicennia marina*. Saad et al. (2010) isolated and cloned the upstream sequences of *ALISAP* translated region (containing the 5'UTR) from the *C₄* halophyte grass *A. littoralis*. The *ALISAP* promoter fused to the *gusA* gene in tobacco showed its expression in an age-dependent, multiple abiotic stress-inducible and tissue-specific manners. The promoter of *Thellungiella halophila* vacuolar H⁺-pyrophosphatase gene (*TsVPI*) was found to have strong activity in almost all tissues except the seeds and its activity was induced by salt stress in leaves and roots, especially in root tips (Sun et al. 2008).

Yin et al. (2002) isolated ~1.2 kb upstream of *BADH* gene from the halophyte *Atriplex centralasiatica* and characterized the basic elements and some to stress-related motifs, such as, the TATA-box, CAAT-box, GC-motif, EIRE, MRE, WUN-motif, heat shock element (HSE), and ABRE. Deletion analysis showed this promoter to be strongly induced by salt stress and identified two salt-responsive enhancer regions localized between -1,115 to -890 bp and -462 to -230 bp. Similarly, Zhang et al. (2008b) isolated upstream region (1,993 bp) of *BADH* gene from *Suaeda liaotungensis* and identified several putative *cis*-elements in the promoter sequence. These two studies suggested that the smallest promoter fragment (-300 to +62 bp) possessed all the essential *cis*-acting elements and was sufficient for NaCl induction. We have also isolated and characterized a promoter (1,875 bp) of an abscisic acid-responsive gene from *Spartina alterniflora* (*SaAsr1*), which was constitutively expressed. In silico analysis of the promoter revealed a number of *cis* regulatory

motifs, such as DRE-CRT (Dehydration Response Element/C-Repeat), ABRE, CBF (C-repeat binding factors), LTRE (low-temperature-responsive element), ERE (Ethylene-responsive element), and LRE (light-responsive element). Our finding suggested that a minimal promoter of 203 bp, along with two *cis*-acting elements (ABRE, ERE), was enough to drive abundant expression of the reporter gene under a variety of abiotic stresses, such as salinity, ABA, and PEG (Baisakh et al. unpublished). Similarly, *in silico* analysis of the promoter (1,295 bp) of a cation transport gene, *SaCTP*, from *Salicornia alterniflora* revealed four ABREs in addition to a number of *cis*-regulating, binding motifs, such as DRE-CRT, CBF, LTRE, E-BOX, and MYB (Subudhi and Baisakh 2011). Comparative analysis of the sequences of *Thellungiella parvula* and *T. salsuginea* with *Arabidopsis thaliana*, showed high conservation of the *SOS1* coding region; however, the promoter regions showed conservation only between the two *Thellungiella* species (Oh et al. 2010).

9.9 “Omics” Approaches to Understand Regulation of Salt Stress Responses in Halophytes

In the post-genomics era, comprehensive analyses using functional genomics technologies such as transcriptomics, proteomics, metabolomics, bioinformatics, and high-throughput DNA sequencing have increased our understanding of the complex regulatory networks associated with stress adaptation and tolerance (Urano et al. 2010). Integrated metabolome and transcriptome analyses showed regulation of many important metabolic pathways at the transcriptional level (Mazzucotelli et al. 2008). On the other hand, many metabolic pathways are regulated at the post-transcriptional level, such as by RNA processing, translational, post-translational regulation, or feedback mechanisms (Kaplan et al. 2007). In addition, metabolites, in addition to their functional roles in stress tolerance, act as signaling molecules (Verbruggen and Hermans 2008).

Application of “omics” approaches in understanding salinity stress tolerance mechanisms in halophytes is an important step towards generating crop varieties that can cope with environmental stresses, and knowledge obtained from the study of different halophytes has provided a wealth of information on stress tolerance mechanisms (Hirayama and Shinozaki 2010). Further, omics-based expansive analysis of the differences between halophytes and glycophytes will provide information on the molecular basis of salinity (and other stresses) tolerance attributes of halophytes.

Microarray-based transcriptomic analysis of *Thellungiella halophila* using *Arabidopsis* cDNA microarray identified high level of constitutive expression of six candidate genes in *T. halophila* (Taji et al. 2004; Gong et al. 2005; Du et al. 2008). Mehta et al. (2005) reported that out of 1,841 EST clones from *Avicennia marina* leaf library, 26 novel genes were highly upregulated under salt stress. Through small-scale transcriptomics approach using suppression subtractive hybridization (SSH), several novel salt-induced genes were identified in *Bruguiera gymnorhiza* (Miyama et al. 2006; Wong et al. 2007; Ezawa and Tada 2009) and *Aegiceras*

corniculatum, where 30 unique ESTs were reported (Fu et al. 2005). Similarly, 27 % of 1,255 ESTs from both leaf and root tissues of *Spartina alterniflora* represented stress response under salinity (Baisakh et al. 2008).

Genome and transcriptome analyses of halophytes by next generation sequencing (NGS), such as Roche 454 and illumina-Solexa RNA-Seq, can reveal pathways and networks, which can provide clues to the stress behavior of extremophiles (Bressan et al. 2013). Recently, a draft genome sequence of *Thellungiella parvula* provided resources for high-resolution genome-wide comparison of its extremophile attributes with its glycophyte relative, *A. thaliana* (Dassanayake et al. 2011). Through NGS, the authors obtained the de novo assembled genome in 1,496 gap-free contigs with a number of tandem duplications that suggested a possible basis for *T. parvula*'s extremophile lifestyle, which experiences highly saline, poor, degraded and toxic soils, and very high temperature extremes (Oh et al. 2012). Similarly, NGS-based transcriptome analysis of *Porteresia coarctata* under different conditions generated a total of 152,367 unique transcript sequences, and their functional annotation revealed that genes involved in diverse cellular processes including amino acid biosynthesis, hormone biosynthesis, secondary metabolite biosynthesis, carbohydrate metabolism and cell wall structures, contributed to its adaptation under high salinity and submergence conditions (Garg et al. 2013). Transcriptome analysis of a dicot recretohalophyte *Reaumuria trigyna* in response to salinity stress using NGS identified 44 Gene Ontology (GO) terms, 119 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and 25 clusters of orthologous groups (COG) families (Dang et al. 2013). Transcription profiles of these genes showed their relation to ion transport and reactive oxygen species scavenging system being overrepresented under salinity, which most likely allow it to tolerate the stress imposed by semi-desert saline soil.

Metabolomics studies of halophytes can improve our understanding of cellular responses to ion intoxication. Researches with different species have suggested that hormonal regulation modulates induction of the physiological responses in non-adapted species under stress, whereas better-adapted species present a basal metabolic configuration that allows them to tolerate environmental cues (Gagneul et al. 2007; Arbona et al. 2010; Pérez-Alfocea et al. 2011). Comparative analysis of *Thellungiella halophila* with *Arabidopsis* revealed contrastingly different metabolome profiles between the two species. *Thellungiella* contained higher levels of several osmolytes, such as fructose, sucrose, complex sugars, malate, and proline in comparison to *Arabidopsis* even under control conditions (Urano et al. 2010).

A major challenge in the "omics" era remains with the interpretation and integration of large datasets. Highly sophisticated systems biology approaches are required to integrate diverse and massive datasets into intelligible models, which will improve our understanding of the gene regulation networks and their biological relevance under certain conditions (Moreno-Risueno et al. 2010). Another major challenge with "omics" approaches in halophytes is the transfer of such knowledge to crop species. Translational research seems feasible and can potentially enable the reestablishment of regulatory pathways and networks in crops, which will open up avenues for new biotechnological applications in the future.

9.10 Conclusion and Future Perspective

The salt-affected areas in the world are increasing every day due to the degradation of the irrigation system, addition of waste salts, and increasing contamination of underground water sources (Ashraf 2002), which necessitates development of salt-resistant crops to sustain food production to meet the demand of ever-increasing world population (Flowers and Yeo 1995). Glycophyte crops, despite variation in their salt tolerance ability, do not complete their life cycle or are severely affected with respect to their growth and productivity under salinity. To achieve success through conventional breeding, we must identify molecular markers associated with salt tolerance traits to reduce the breeding cycles in development of salt-tolerant crops. Moreover, very few germplasm in the primary gene pool of food crops are available that can reproduce effectively at high salt concentration. While germplasm are available within the secondary gene pool with high salt tolerance, crossbreeding using these resources has not been very successful.

Tertiary gene pool, on the other hand, has several potential species, such as halophytes that have demonstrated, as “salt loving” plants, their capability of thriving under extremely high salinity, thus providing suitable germplasm for identifying and developing new crop systems for saline agriculture (Shabala 2013). One approach is to domesticate halophytes in areas unsuitable for crop establishment. The other approach is to develop salt-tolerant cultivars of conventional crops by introducing halophyte genes directly (Bohnert and Jensen 1996). There are several transgenics that have been developed with halophyte genes and showing high salt tolerance. But, commercial transgenic plants should retain high productivity along with other important traits for agriculture. Hence, transgenic breeding requires identification of stress-related genes and their related promoters.

Despite considerable progress, our understandings of the processes underlying the visible appearances of salt tolerance and salt adaptation of halophytes are still far from complete. There is still a lot to characterize about halophytes and the diversity of mechanisms they employ to cope with salinity. Salt tolerance and adaptation are complex phenomena, which involve multiple physiological, and biochemical mechanisms and numerous genes. Some of the basic questions that need to be addressed are: how halophytic plants discriminate between Na^+ and K^+ ; how they regulate Cl^- transport; the energetics and regulation of ion transport and compartmentation; the influence of O_2 supply on transport; importance of transpiration in ion accumulation; and control of relative water potential through stomatal aperture, etc. (Colmer and Flowers 2008). To answer these questions, we need to use comparative physiology coupled with genomic, proteomic, and metabolomic approaches that should yield gene regulatory networks and novel stress modules. No single halophyte can be a universal model to address the afore-mentioned questions. Therefore, choosing a right model halophyte is essential in understanding unique genes associated with tolerance/adaptation mechanisms in monocot and dicot halophytes; these may lead to their direct translation into monocot or dicot crops. Fortunately, there are halophyte relatives reported for several crops.

Therefore, research efforts should be made to produce interspecific hybrids, which can serve as genetic resources for mapping of salt tolerance traits and development of crop plants introgressed with halophyte chromosome segments. Concerted efforts must integrate genetic engineering with marker-assisted selection of stress-related genes and QTLs to substantially improve stress tolerance in crop plants. This calls for an international collaboration among research community to identify and exploit need-based halophyte resources as our future generations will rely upon these to a larger extent than now, especially under the inexorable climate change scenarios.

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