

Review

Role of sodium and hydrogen (Na^+/H^+) antiporters in salt tolerance of plants: Present and future challenges

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Salt tolerant plants have been characterized by their ability to cope with osmotic and ionic stresses caused by elevated sodium chloride (NaCl) concentrations. For homeostatic control of Na^+ , plants have evolved a system of membrane channels and antiporters that facilitate the influx and efflux of sodium (Na^+) ions at the roots and establish a steady state rate of entry of Na^+ into the plant, compartmentation of Na^+ into the cell vacuoles and transfer to various plant tissues. To enhance the salt tolerance of salt sensitive plants, genetic engineering with sodium and hydrogen (Na^+/H^+) antiporters is one of the preferred methods in recent years. The aim of this study is to highlight and discuss the recent progress in understanding Na^+ transport in plants, and genetic engineering of plants with Na^+/H^+ antiporters to increase their salt stress tolerance. The present status of salt stress tolerance in transgenic plants was examined and possibilities were recommended that may further enhance salt tolerance if given proper research attention. Moreover, future challenges including environmental risk assessment confronting transgenic plants transformed with Na^+/H^+ antiporters were also discussed.

Key words: Salt tolerance, Na^+/H^+ antiporters, transgenic plants, vacuolar compartmentalization.

INTRODUCTION

Salinity is one of the major abiotic stresses that adversely affect plant growth and productivity (Munns, 2005). About 20% of irrigated land under agricultural practices is adversely affected by salinity (Flowers and Yeo, 1995). The salinity problem in agriculture is further aggravated because of the use of poor quality water for irrigation. Plant responses to salt stress are partly governed by accumulation of compatible solutes and expression of certain stress related genes that help to cope with osmotic stress (Chinnusamy et al., 2005). The most important aspect of plant responses leading to salt stress tolerance is the regulation of uptake and distribution of Na^+ ions (Tester and Davenport, 2003). Along osmotic homeostasis, maintenance of ionic homeostasis is an important strategy for achieving enhanced tolerance to environmental stresses (Sun et al., 2009). Ionic homeostasis is based on the excretion of Na^+ ions out of the roots or storing in the vacuoles. On the basis of coping excess Na^+ in the cytosol, plants can be classified into two major groups i.e. halophytes and glycophytes (Khan and Duke, 2001). Most of the crop plants are glycophytes that restrict the intake

of Na^+ into the cytoplasm. However due to the electrochemical gradient across the cell membrane, Na^+ influx can not be completely prevented (Hasegawa et al., 2000). Plants have developed specific proteins and channels to facilitate ionic movements across cellular and vacuolar membranes in the roots and leaf cells (Blumwald, 2000). Plants tolerant to saline stress have been found with highly developed and efficient transport system of cell membrane and vacuolar antiporter proteins that control and restrict the intake of ions to root cells or confine them inside vacuoles (Shi et al., 2003).

In Arabidopsis, various transporters of monovalent cations, such as HKT1, members of the NHX family, and salt overly sensitive 1 (SOS1), have been shown to play important role in regulating movement and distribution of Na^+ ions (Shi et al., 2003; Rus et al., 2004; Pardo et al., 2006). These Na^+/H^+ antiporters play a crucial role in maintaining cellular sodium level, cytoplasmic pH and cell turgor (Serrano et al., 1999). They have been isolated from various plant species including Arabidopsis, rice, *Atriplex gmelini* and *Mesembryanthemum crystallinum*

(Fukuda et al., 1999; Chauhan et al., 2000; Shi et al., 2000). In recent years, transformation of various plant species with Na⁺/H⁺ antiporters has resulted in enhanced salt stress tolerance and growth improvement (Yokoi et al., 2002; Yoshida, 2002; Ashraf and Akram, 2009).

Despite much progress towards achieving salt tolerance with Na⁺/H⁺ antiporters in transgenic plants, several issues are arising that needs proper research attention in order to fully exploit the potential of genetic transformation of crop plants with Na⁺/H⁺ antiporters, enhancing their yield capacity under natural saline conditions. The future deployment of these salt tolerant transgenic plants to potential receiving environments for commercial cultivation will need assessment of potential adverse effects on the environment, based on scientifically sound risk assessment procedures. The present study focuses on the role of various membrane transport proteins in transgenic plants in relation to salt stress tolerance and plant growth characteristics. We also tried to discuss environmental risk assessment issues that these salt tolerant transgenic plants will confront in the future.

THE SOS REGULATORY PATHWAY

Understanding of plant responses to excessive Na⁺ concentration has been progressed since the discovery of the Salt Overly Sensitive 1 (SOS1) pathway in Arabidopsis (Shi et al., 2000). The prominent genes working in this pathway include SOS1, a plasma membrane Na⁺/H⁺ antiporter (Shi et al., 2000), SOS2, a serine/threonine protein kinase (Liu et al., 2000), SOS3, a Ca²⁺ sensor (Liu and Zhu, 1997; Zhu et al., 1998), HKT1, a transporter that facilitate Na⁺ uptake into the cell and NHX1, a Na⁺/H⁺ antiporter localized on vacuolar membrane for Na⁺ compartmentation in the vacuole. The SOS mutant loci were identified as important salt tolerance determinants in Arabidopsis by screening for reduced root bending of seedlings grown in the presence of sodium (Wu et al., 1996). The SOS mutants showed specific hypersensitive to Na⁺ and Li⁺ transport. These mutants also exhibited growth deficiencies on media containing low potassium concentrations (Horie and Schroeder, 2004). The SOS1 gene encodes a Na⁺/H⁺ antiporter that has 12 transmembrane domains in the N-terminal half and a long hydrophilic C-terminal tail (Shi et al., 2000). Further, the various SOS mutants were categorized into five groups or five loci i.e. SOS1, SOS2, SOS3, SOS4 and SOS5 (Wu et al., 1996; Liu and Zhu, 1997; Zhu et al., 1998; Zhu, 2000).

Sodium influx and Na⁺/K⁺ balance

Under normal physiological conditions, plants maintain a high potassium and sodium (K⁺/Na⁺) ratio in the cytosol (Higinbotham, 1973). Accumulation of high Na⁺ concentration in the extracellular spaces generates a very

high electrical membrane potential difference, which facilitate a passive movement of Na⁺ ions into the cytosol. This passive movement of Na⁺ ions into root cells is facilitated by ion transporters or channels. These include the high affinity K⁺ transporter HKT1, LCT1, and other non-selective cation channels (CNGCs and GLRs) (Figure 1) (Apse and Blumwald, 2007). These different transporters function in parallel, mediating Na⁺ uptake into the roots and are dependant upon species and growth conditions. Transporters of the HKT1 family function as a symporter for both Na⁺ and K⁺ and as a selective Na⁺ uniporter (Garcia-deblas et al., 2003). The HKT1 transporters have been extensively studied in Arabidopsis and rice. Arabidopsis contains only a single HKT1 gene that was found in various cell types. However, insertional inactivation of Arabidopsis HKT1 showed no significant changes in the Na⁺ influx, and it was suggested that this gene does not play a major role in Na⁺ influx (Essah et al., 2003).

However, in rice, several members of the HKT1 family have been identified that play a significant role in the Na⁺ influx (Kader et al., 2006). The tissue specific activity of HKT1 has recently been shown to correlate with Na⁺ movement from root to shoot and the subsequent salt tolerance. Transgenic Arabidopsis expressing the HKT1 gene in the mature root stele cells showed a drastic decrease of Na⁺ accumulation in the shoot by 37 to 64% (Moller et al., 2009). This decrease in shoot Na⁺ content was mediated by the increased Na⁺ influx into the root stele cells, which in turn, decreased the flow of Na⁺ from root to shoot and increased salt tolerance. On the contrary, the constitutive expression of HKT1 on the whole plant level resulted in high accumulation of Na⁺ in roots as well as shoots and the transgenic plants showed poor shoot growth. The authors concluded that the tissue specific modification of a Na⁺ transport process can reduce shoot Na⁺ accumulation that results in salt tolerance.

Apart from the HKT1, some other low affinity cation transporters were investigated in several studies. A low affinity Na⁺ transporter, LCT1, was cloned from wheat (Schachtman et al., 1997). In yeast cells, the LCT1 was found to mediate uptake of some other ions such as Na⁺, rubidium (Rb⁺), calcium (Ca²⁺), lithium (Li⁺) and caesium (Cs⁺) (Amtman et al., 2001). The molecular determinants of non-selective cation channels are still not fully explored. Recently, two families of non-selective cation channels, cyclic nucleotide-gated channels (CNGCs) and glutamate-activated channels (GLRs) have been identified, which play an important role in root Na⁺ uptake (Leng et al., 2002; Tester and Davenport, 2003; Demidchik et al., 2004; Maathuis, 2007). At least five members of this gene family (CNGC1, 2, 3, 4 and 10) have been characterized from Arabidopsis (Apse and Blumwald, 2007). Some of these AtCNGCs (CNGCs 1, 3 and 4) are permeable to both Na⁺ and K⁺ (Leng et al., 2002; Balague et al., 2003; Gobert et al., 2006). While the CNGC2 and CNGC10 were found to have permeability to

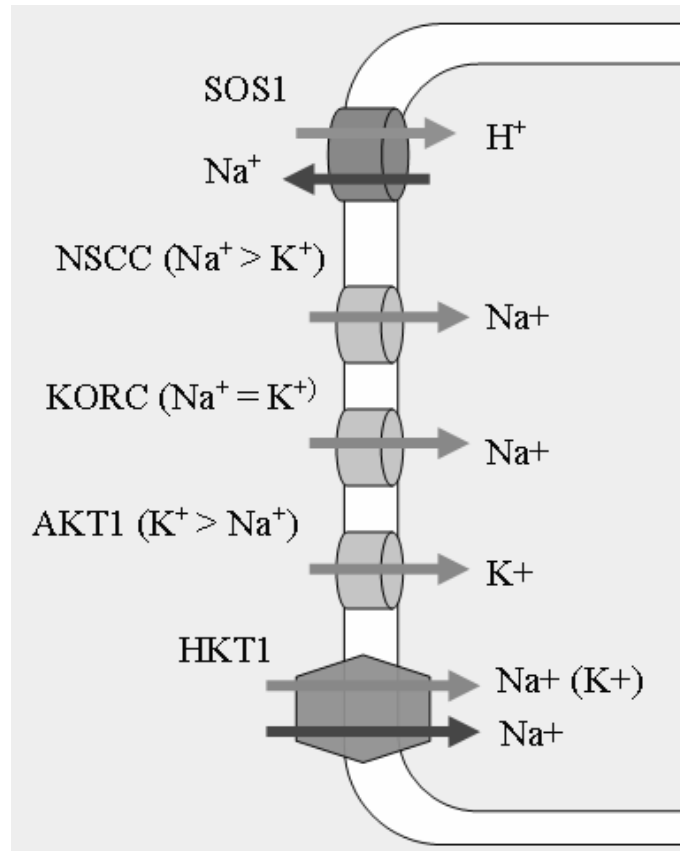


Figure 1. Schematic representation of Na^+ influx and maintenance of Na^+/K^+ balance. Various specific and non-specific cation channels are involved in sodium and potassium movement across the plasma membrane. High Na^+ concentration in the extracellular environment is taken inside the cytosol through the high Na^+ affinity HKT1 antiporter and the non-selective cation channels (NSCC) and the outward rectifying K^+ channels (KORC) (modified from Yamaguchi and Blumwald (2005)).

K^+ only (Li et al., 2005). The AtCNGC3 is involved in short term Na^+ uptake in plant roots that contributes some impact on plant salt tolerance (Gobert et al., 2006), while AtCNGC10 has been reported to contribute to Na^+ uptake and long distance transport (Guo et al., 2008). Moreover, transcriptomic studies conducted in Arabidopsis have revealed significant regulation of other CNGCs isoforms in response to salt stress (Maathuis, 2006). In addition, the ionotropic glutamate receptors (GLRs) have also been suggested to have a role in Na^+ uptake (Demidchik et al., 2004).

Sodium efflux

In the presence of high concentration of Na^+ inside and outside of the cell, the electrochemical gradient that is generated across the membrane makes an active transport of Na^+ out of the cell (Blumwald, 2000). In microorganisms and animals, this active transport is

facilitated by ATP hydrolysis. For this purpose, specific Na^+ -ATPases are present in the plasma membrane. No such type of Na^+ pumps have been found in plants (Horie and Schroeder, 2004). The only channels through which Na^+ is excluded of the cell are Na^+/H^+ antiporters. In plants, various Na^+/H^+ antiporters have been classified into three families; CPA1 (NHX, eight members), NhaD (two members), and CPA2 (including CHX, twenty eight members and KEA, six members) (Sze et al., 2004). Among the eight members of the CPA1 family, two members (AtNHX7/SOS1 and AtNHX8) have been identified as localized on the plasma membrane (Shi et al., 2003; An et al., 2007). AtNHX7/SOS1 is a Na^+/H^+ antiporter, while AtNHX8 has been characterized as Li^+/H^+ antiporter (An et al., 2007). The other six members (AtNHX1 to 6) are vacuolar/endosomal antiporters (Pardo et al., 2006).

The SOS1 was first identified in an Arabidopsis mutant that showed altered root growth on saline media (Wu et al., 1996). Another two genes (SOS2 and SOS3) were

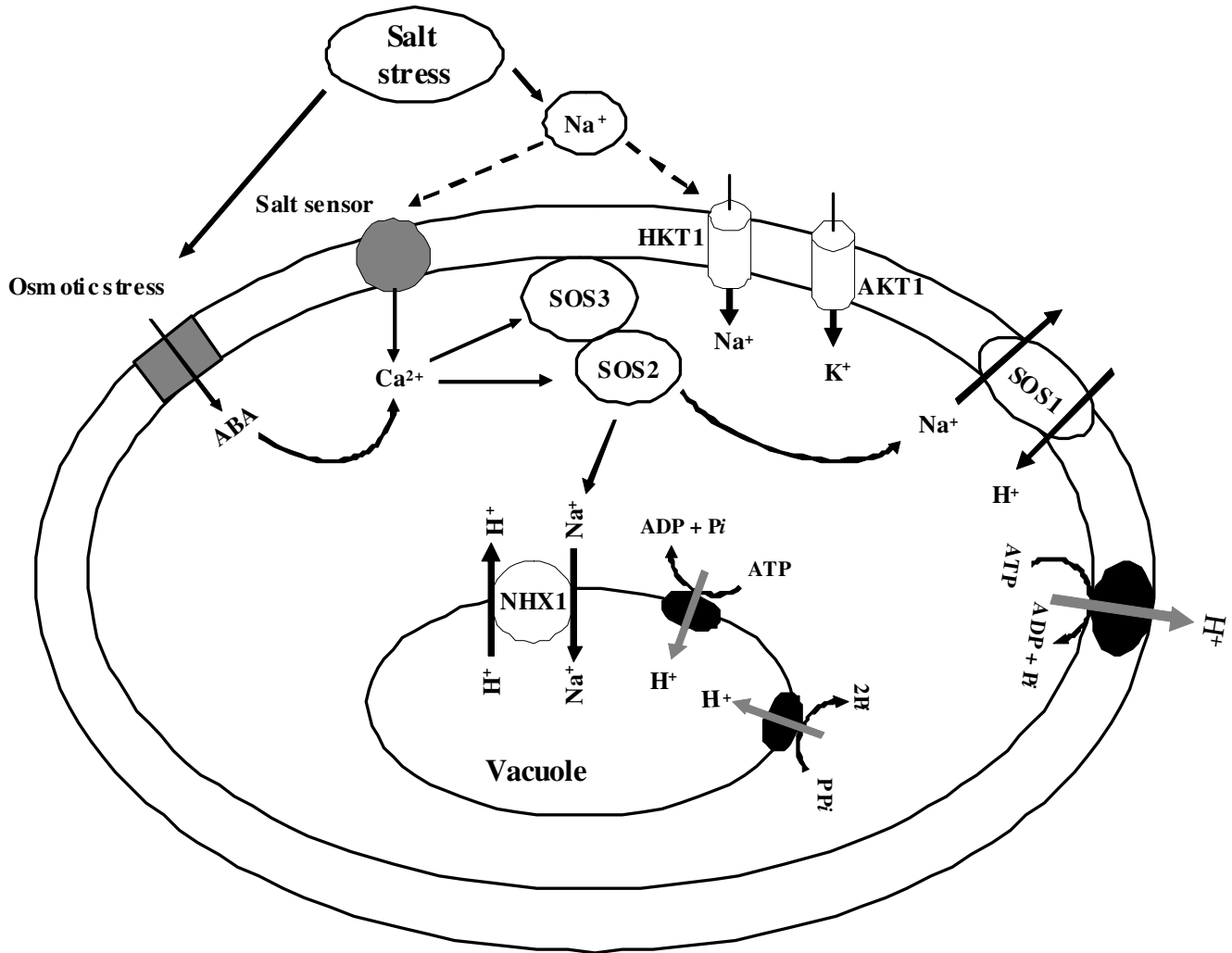


Figure 2. Schematic representation of the SOS signaling pathway for ion homeostasis in Arabidopsis. Electrochemical gradient is generated through the H^+ -ATPase in the plasma membrane and H^+ -PPase in the vacuolar membrane. The extracellular salt stress signals are perceived by SOS3 through the Ca^{2+} signals. The SOS3 activates SOS2, which then phosphorylates the SOS1, a plasma membrane Na^+/H^+ antiporter that transport Na^+ out of the cytosol. SOS2 also phosphorylates the vacuolar Na^+/H^+ antiporter, which accumulates Na^+ inside the vacuole (modified from Chinnusamy et al. (2005)).

also identified that function as regulators for the activity of SOS1 and enhances the SOS1 mediated Na^+/H^+ exchange across plasma membrane (Qiu et al., 2003). Mutation of the SOS1 gene in Arabidopsis resulted in loss of the Na^+/H^+ exchange activity across the plasma membrane (Qiu et al., 2003). The authors reported that the mutant Arabidopsis retained some Na^+/H^+ activity, which was due to some other cation/proton exchangers. However, this activity was only observed in membranes isolated from leaves, and root cell membranes were not analysed in that study. The role of the SOS genes in salt tolerance of plants has been evaluated in several studies. Yang et al. (2009) generated several transgenic Arabidopsis lines that expressed SOS1, SOS2, SOS3 and *AtNHX1*, either individually or in combination. They reported high salt stress tolerance for transgenic plants

with either SOS1 or SOS3, while the *AtNHX1* expression alone did not increase stress tolerance.

Sodium compartmentalization

Besides excluding excess Na^+ from cytosol, the vacuolar compartmentalization of Na^+ is another very important mechanism to cope with salinity stress (Apse and Blumwald, 2002). Excess Na^+ in the cytosol is taken into the vacuole by cation/ H^+ antiporters localized on vacuolar membranes (Figure 2). These antiporters are driven by electrochemical gradient of protons generated by the vacuolar H^+ -translocating enzymes such as H^+ -ATPase and H^+ -PPase (Apse and Blumwald, 2007). The vacuolar Na^+/H^+ antiporters have been extensively studied in

Arabidopsis. The organization, physiological, expression pattern and structure-function properties of these vacuolar antiporters have previously been reviewed (Mayeshima, 2001; Isayenkov et al., 2010). Various vacuolar antiporters such as AtNHX1 to 6 have been cloned from Arabidopsis (Gaxiola et al., 1999). Since then, such types of vacuolar antiporters have been identified in a wide range of plant species (Pardo et al., 2006). Among the six vacuolar antiporters, AtNHX1, AtNHX2 and AtNHX5 have been characterized as functional Na⁺/H⁺ antiporters (Yokoi et al., 2002). Li et al. (2009) conducted functional characterization of AtNHX4, another member of the vacuolar antiporters. Unlike AtNHX1, functional inactivation of AtNHX4 in Arabidopsis showed enhanced tolerance to salt stress, lower Na⁺ content and high K⁺ content under high salt stress compared with wild-type plants. The authors further postulated that unlike AtNHX1 which brings Na⁺ inside the vacuolar lumen, AtNHX4 transports Na⁺ out of vacuolar lumen to the cytosol.

In several studies, the role of vacuolar antiporters in Na⁺/K⁺ homeostasis, pH regulation and overall plant salt stress tolerance has been explained (Yamaguchi et al., 2001; Apse et al., 2003). It was reported that during exposure to saline condition, various crop plants up-regulate the expression of genes encoding NHX like antiporters, which in turn, play important role in the salt tolerance of these plants. The expression of NHX genes was up-regulated by salinity in cotton (Wu et al., 2004), rice (Fukuda et al., 1999) and wheat (Saqib et al., 2005). The genetic engineering of crop plants with vacuolar antiporters and the resultant salt tolerance was therefore discussed.

GENETIC ENGINEERING OF PLANTS WITH ION TRANSPORTERS TO CONFER SALT STRESS TOLERANCE

Transformation of several plant species with ion transporters, particularly the vacuolar Na⁺/H⁺ antiporter genes, have resulted in enhanced tolerance to elevated salt concentrations. These transgenic plants are listed in Table 1. Transgenic Arabidopsis plants were generated by over expressing the plasma membrane Na⁺/H⁺ antiporter, *AtSOS1* gene under *CaMV35S* constitutive promoter (Shi et al., 2003). Upon salt stress, transgenic plants up-regulated the *SOS1* transcript level that resulted in lower Na⁺ accumulation in root, xylem stream and shoot cells. Moreover, transgenic lines exhibited improved germination rate, chlorophyll content, and protein level and reduced accumulation of shoot Na⁺ content. More importantly, about 11 to 15.4% increase in root growth was reported. Transgenic Arabidopsis transformed with *MsNHX1* from alfalfa showed improved germination and seedling growth under salt stress (Bao-Yan et al., 2008). Brini et al. (2007) transformed Arabidopsis with the wheat vacuolar Na⁺/H⁺ antiporter (*TNHX1*) and the resultant

plants showed improved growth under 200 mM NaCl concentration, while the control plants showed chlorosis and growth inhibition. Transgenic plants also exhibited tolerance to water deprivation. Similarly, introduction of the Na⁺/H⁺ antiporter from *Suaeda salsa*, referred as *SsNHX1* in Arabidopsis conferred tolerance to salt (200 mM NaCl) and cold stress (7°C) (Li et al., 2007). Transgenic plants showed normal growth, while the non-transgenic plants were found with severe growth retardation under salt stress. The Arabidopsis vacuolar membrane Na⁺/H⁺ antiporters have been introduced in several crop plants to enhance their salt tolerance. Introduction of *AtNHX1* in brassica conferred salt stress tolerance and transgenic plants showed 2.3% high plant fresh weight and 2.34% high grain yield under (10 mM) NaCl (Zhang et al., 2001). Transgenic cotton that expressed Arabidopsis *AtNHX1* showed enhanced salt stress tolerance (He et al., 2005).

Among the six different Na⁺/H⁺ antiporters isolated from Arabidopsis, only *AtNHX1*, *AtNHX2* and *AtNHX5* were reported as functional Na⁺/H⁺ antiporters and were functionally characterized. These genes showed high expression in seedlings roots and shoots. However, the physiological role of *AtNHX3* was not known until recently. Liu et al. (2008) reported constitutive expression of *AtNHX3* in sugar beet (*Beta vulgaris*). Transgenic plants exhibited high salt tolerance and accumulated high content of potassium ions in roots and storage roots in the presence of 300 or 500 mM NaCl. Apart from salt tolerance, transgenic sugar beet also accumulated high sugar content in storage roots. This finding has demonstrated the great potential of this gene in improving the yield and quality of crop plants in the near future.

Transformation of tobacco with *GhNHX1* from cotton conferred salt tolerance and transgenic plants showed about 100% increase in plant dry weight (Wu et al., 2004). In another experiment, transgenic tobacco transformed with brassica *BnNHX1* showed improved plant growth and better seed production under 200 mM NaCl stress, while wild-type plants died (Wang et al., 2004). In some other experiments, transgenic tobacco with *HbNHX1*, *SynnhaP1* and *AtNHX1* conferred salt stress tolerance (Lu et al., 2005; Hossain et al., 2006; Soliman et al., 2009). More importantly, transgenic tobacco with vacuolar Na⁺/H⁺ antiporter (*AINHX1*) from *Aeluropus littoralis* showed about 150% increase in plant dry weight under salt stress (Zhang et al., 2008). The expression of *AgNHX1* in transgenic rice plants exhibited enhanced survival of seedlings (81 to 100%) under salt stress (Ohta et al., 2002). Similarly, transformation of rice with *OsNHX1* and *PgNHX1* conferred salt tolerance and transgenic plants showed higher shoot and root growth (Fukuda et al., 2004; Chen et al., 2007; Verma et al., 2007). Transformation of wheat and maize with *AtNHX1* showed tolerance to salt stress (Xue et al., 2004; Yin et al., 2004). Transgenic wheat exhibited increase in shoot and root dry weight, while transgenic maize showed improved

Table 1. List of abiotic stress tolerant transgenic plants with Na⁺/H⁺ antiporters and related genes.

Target	Transgene	Host	Tolerance	Growth improvement	Reference
<i>Arabidopsis thaliana</i>	<i>AtNHX1</i>	<i>Arabidopsis thaliana</i>	salt	Improved growth under 200 mM NaCl	Apse et al., 1999
<i>Arabidopsis thaliana</i>	<i>AtSOS1</i>	<i>Arabidopsis thaliana</i>	salt	11 to 15% increase in root growth	Shi et al., 2003
<i>Arabidopsis thaliana</i>	<i>TNHX1, TVP1</i>	wheat	salt, drought	Improved shoot growth	Brini et al., 2007
<i>Arabidopsis thaliana</i>	<i>SsNHX1</i>	<i>Suaeda salsa</i>	salt, freezing	Normal plant growth at 200 mM NaCl and at -7 °C	Li et al., 2007
<i>Arabidopsis thaliana</i>	<i>LeNHX2</i>	<i>Solanum lycopersicum</i>	salt	Better growth and high fresh weight	Rodriguez-Rosales et al., 2008
<i>Arabidopsis thaliana</i>	<i>MsNHX1</i>	Alfalfa	salt	Better germination and seedling growth	Bao-Yan et al., 2008
<i>Arabidopsis thaliana</i>	<i>GhNHX1, ScNHX1, AtNHX1, SsNHX1, TaNHX1</i>	<i>Arabidopsis</i> , cotton, <i>Suaeda salsa</i> , wheat, yeast	salt, drought	High salt tolerance and photosynthetic activity. Growth performance under salt stress was ranked as <i>GhNHX1</i> > <i>ScNHX1</i> > <i>AtNHX1</i> > <i>SsNHX1</i> > <i>TaNHX1</i>	Liu et al., 2010
<i>Agrostis stolonifera</i> (bentgrass)	<i>AVP1</i>	<i>Arabidopsis thaliana</i>	salt	High biomass content under salt stress	Li et al., 2010
<i>Beta vulgaris</i>	<i>AtNHX3</i>	<i>Arabidopsis thaliana</i>	salt	Increased yield of storage roots	Liu et al., 2008
<i>Brassica napus</i>	<i>AtNHX1</i>	<i>Arabidopsis thaliana</i>	salt	Improved fresh weight and grain yield	Zhang et al., 2001
<i>Brassica juncea</i>	<i>pgNHX1</i>	<i>Pennisetum glaucum</i>	salt	Retained normal plant growth and seed yield at 300 mM NaCl	Rajagopal et al., 2007
<i>Gossypium hirsutum</i>	<i>AtNHX1</i>	<i>Arabidopsis thaliana</i>	salt	Improved fiber yield under 200 mM NaCl	He et al., 2005
<i>Gossypium hirsutum</i>	<i>AVP1</i>	<i>Arabidopsis thaliana</i>	Salt, drought	Improved fibre yield under field condition	Pasapula et al., 2011
<i>Nicotiana tabacum</i>	<i>GhNHX1</i>	<i>Gossypium hirsutum</i>	salt	About 100% increase in dry weight	Wu et al., 2004
<i>Nicotiana tabacum</i>	<i>BnNHX1</i>	<i>Brassica napus</i>	salt	Better seed production	Wang et al., 2004
<i>Nicotiana tabacum</i>	<i>HbNHX1</i>	<i>Hordeum brevisubulatum</i>	salt	Increased dry weight	Lu et al., 2005
<i>Nicotiana tabacum</i>	Nhap type Na ⁺ /H ⁺ antiporter	<i>Synechocystis</i>	salt	Increased biomass and seed production	Hossain et al., 2006
<i>Nicotiana tabacum</i>	<i>AINHX1</i>	<i>Aeluropus littoralis</i>	salt	More Na ⁺ accumulation in roots. High K ⁺ /Na ⁺ ratio in shoots. About 150% increase in dry weight/plant	Zhang et al., 2008
<i>Nicotiana tabacum</i>	<i>AtNHX1</i>	<i>Arabidopsis thaliana</i>	salt	-	Soliman et al., 2009
<i>Nicotiana tabacum</i>	<i>AVP1</i>	<i>Arabidopsis thaliana</i>	Salt, drought	-	Ibrahim et al., 2009
<i>Oryza sativa</i>	<i>AgNHX1</i>	<i>Atriplex gmelini</i>	salt	Increased survival of seedling	Ohta et al., 2002
<i>Oryza sativa</i>	<i>OsNHX1</i>	<i>Oryza sativa</i>	salt	Maintained growth at 200 mM NaCl	Fukuda et al., 2004
<i>Oryza sativa</i>	<i>SOD2</i>	Yeast	salt	High shoot weight	Zhao et al., 2006
<i>Oryza sativa</i>	<i>OsNHX1</i>	<i>Oryza sativa</i>	salt	Delayed flowering and growth retardation	Chen et al., 2007
<i>Oryza sativa</i>	<i>PgNHX1</i>	<i>Pennisetum glaucum</i>	salt	Improvement in shoot and root length	Verma et al., 2007
<i>Petunia hybrida</i>	<i>AtNHX1</i>	<i>Arabidopsis thaliana</i>	salt, drought	-	Xu et al., 2009
Tall fescue	<i>AtNHX1</i>	<i>Arabidopsis thaliana</i>	salt	Improved shoot and root dry weight	Zhao et al., 2007
<i>Triticum aestivum</i>	<i>AtNHX1</i>	<i>Arabidopsis thaliana</i>	salt	Increase in shoot and root dry weight	Xue et al., 2004
<i>Zea Mays</i>	<i>AtNHX1</i>	<i>Arabidopsis thaliana</i>	salt	Improved germination	Yan et al., 2004
<i>Zea Mays</i>	<i>OsNHX1</i>	<i>Oryza sativa</i>	salt	Increased biomass production	Chen et al., 2007

‘-’ Means information is not available.

germination. Recently, transgenic maize plants were generated that expressed the *OsNHX1* from rice (Chen et al., 2007). Transgenic plants accumulated high biomass in the presence of 200 mM NaCl concentration. Tomato plants over expressing vacuolar *AtNHX1* showed improved growth, flower and seed production under high salt concentration (200 mM NaCl) (Zhang and Blumwald, 2001). HAL1, a K⁺ regulator in *Saccharomyces cerevisiae* was expressed in melon and tomato and the transgenic plants showed improved tolerance to salt stress (Bordas et al., 1997; Gisbert et al., 2000).

Moreso, several proton pumps in the cell and vacuolar membranes such as plasma membrane H⁺-ATPase, the vacuolar-type H⁺-ATPase and the vacuolar H-pumping pyrophosphatase are known for their role in generation of proton gradient and ion transport system in plants (Barkla and Pantoja, 1996; Sondergaard et al., 2004). A vacuolar H⁺-PPase pump encoded by *AVP1* gene was expressed in Arabidopsis and its expression conferred salt and drought tolerance (Gaxiola et al., 2001). Transgenic Arabidopsis plants performed better than the wild-type plants, particularly under limited water condition. The *AVP1* gene was further studied in transgenic tomato where its over-expression improved root biomass under water stress condition (Park et al., 2005). Under limited water condition, the product of the *AVP1* gene stimulates auxin transport in the root system and leads to larger root systems, which enables the plants to absorb water more efficiently (Pasapula et al., 2011). In one study, He et al. (2005) transformed tobacco with *AtNHX1* under *CaMV35S* promoter. The authors reported improved salt tolerance in transgenic plants as compared to control plants under greenhouse condition. More importantly, the transgenic plants also exhibited salt tolerance in the field (US applications 04-145-04N and 05-066-06N). As compared to control, transgenic plants showed improved root growth, plant height, and photosynthetic rate under salt stress. The most significant finding of this study was increase in cotton yield in transgenic lines. An average of 25% increase in boll numbers and fiber yield per line was reported. As recommendation, the authors suggested that although the salt tolerance is higher in transgenic lines as compared to controls, it is limited by the capacity of the proton pumps. Therefore, simultaneous over-expression of the vacuolar Na⁺/H⁺ antiporter and vacuolar H⁺ pump will be a good strategy to further increase salt tolerance in transgenic plants. In more recent studies, the importance of the vacuolar *AVP1* gene encoding H⁺-pyrophosphatase in salt and drought tolerance has been demonstrated in Arabidopsis (Brini et al., 2007), and in cotton (Pasapula et al., 2011). In the later study, transgenic cotton plants were generated with Arabidopsis *AVP1* gene. Transgenic cotton plants were observed with improved salt and drought tolerance under greenhouse conditions. Under dry land conditions in the field, transgenic plants produced 20% higher fiber yield than that of wild-type plants. This study revealed that the *AVP1* gene alone is sufficient to

increase cotton yield as much as that previously reported in *AtNHX1* cotton plants under field condition by He et al. (2005). In conclusion, Pasapula et al. (2010) suggested that the *AVP1* gene has the potential to increase both salt and drought tolerance of plants in areas where water and salinity are the major limiting factors for agricultural productivity. Further, they recommended that the *AVP1* gene should be expressed in other economically important crop plants either alone or with the vacuolar Na⁺/H⁺ antiporters to enhance their drought and salt tolerance.

In addition to the extensive use of both cellular and vacuolar membrane Na⁺/H⁺ antiporters in transgenic plants, other genes working in Na⁺ transport across cellular membrane should be utilized to enhance salt tolerance of plants. The Na⁺ transporter HKT1 is one of such genes. Originally, the HKT1 was considered to be a high affinity K⁺ transporter but later it was discovered that this is also involved in Na⁺ influx. The HKT1 gene, if expressed in specific roots cells may reduce Na⁺ transport to shoots. Accumulation of excess Na⁺ in shoot cells negatively affects cellular and physiological processes vital for plant growth and survival. The strategy to restrict or reduce Na⁺ transport from roots to shoots by accumulation at the root level in specific cells may increase salt tolerance of plants. In one study, Moller et al. (2009) reported cell type-specific expression of HKT1;1 in the mature root stele cells in Arabidopsis. This cell type-specific expression of HKT1;1 resulted in high Na⁺ accumulation in the root stele cells that, in turn, significantly reduced root to shoot Na⁺ transport. Transgenic plants with reduced Na⁺ content in shoots exhibited enhanced salt stress tolerance. On the contrary, when the HKT1;1 was constitutively expressed under the *CaMV35S* promoter. Transgenic plants accumulated high Na⁺ content in shoots and showed poor plant growth. In conclusion the authors suggested that cell type-specific gene expression of HKT1;1 is an important strategy to reduce Na⁺ content in the transpiration stream resulting lower accumulation in shoots and high salt tolerance.

The aforementioned examples of successful transformation of several plant species with Na⁺/H⁺ antiporters and achievement of stress tolerance therefore indicated the usefulness of this approach for enhancing salt stress tolerance of crop plants. In particular, engineering transgenic plants with the ability to confine excess Na⁺ in vacuoles seems to be the best strategy to cope with increasing soil and water salinity.

ENVIRONMENTAL SAFETY OF SALT TOLERANCE-INDUCING Na⁺/H⁺ ANTIPORTERS

To cope with excessive soil and water salinity and to enable crop plants to perform better under such conditions, the role of various membrane and vacuolar antiporter proteins has been extensively studied.

Transgenic plants with antiporter genes are in the process of development and in the near future will be deployed for commercial cultivation in areas where salinity is the major problem. Nevertheless, the success of these salt tolerant transgenic plants with antiporters, in the long run depends on the safety of these genes to environmental entities and biodiversity preservation.

Environmental risk assessment is the process that identifies and evaluates the potential adverse effects of transgenic plants with introduced genes and their corresponding traits on the environment, taking also into account risks to human health (Raybould, 2006). Typically, the risk assessment process follows four major steps; 1) Hazard identification or problem formulation in case of transgenic plants; 2) exposure assessment; 3) effects assessment and 4) risk characterization (Andow and Zwahlen, 2006; Nickson et al., 2008). The current risk assessment procedures of transgenic plants are based on the principles and guidelines of the Cartagena Protocol on Biosafety at the Convention on Biological Diversity. These procedures have already been used to assess the potential adverse effects of first generation transgenic plants with insect resistance, herbicide tolerance and virus resistance traits. Transgenic plants with abiotic stress tolerance genes are in the developmental process and have not been commercialized till date. Some of the transgenic plants with salt and drought tolerance traits are under field trials for risk assessment studies (OGTR, 2005; OGTR, 2008).

Despite the different and complex nature of abiotic stress tolerance traits, there has been a general agreement in the scientific community that these plants will use the same basic risk assessment procedures currently in practice for insect resistance or herbicide tolerance traits. However, there is a need to investigate and search for additional considerations in the risk assessment process. In case of transgenic plants with antiporter genes that confer salt stress tolerance, the following points would elaborate whether or not additional elements in risk assessment are required and which points should be focused in the risk assessment.

First, unlike insect resistance and herbicide tolerance traits, the incorporation of salt tolerance into crop plants through genetic engineering is not a novel activity. Conventional breeding techniques have long been used to develop salt tolerant crop varieties. In the 20th century, using conventional methodologies, plant breeders used and exploited the available genetic variation at the intra-specific, inter-specific and inter-generic levels to develop salt tolerant crop plants (Ashraf and Akram, 2009). Using these conventional methods, salt tolerance of several crop plants such as rice, wheat and other crop plants was improved (Colmer et al., 2006; Singh and Sharma, 2006; Singh et al., 2007). Although limited success was achieved with conventional methods, some salt tolerant lines such as those of alfalfa (Al-Doss and Smith, 1998; Dobrenz, 1999) and bread wheat (Ashraf

and O'leary, 1996; Hollington, 2000) were tested under natural salt affected soils. The role of Na⁺/H⁺ antiporters in these conventionally bred crop varieties is obvious due to their major role in maintaining ionic and homeostatic balance when plants are exposed to elevated levels of salt stress. The aforementioned conventional approaches for developing salt tolerant crop varieties might also have involved Na⁺/H⁺ antiporter genes along with introduction of other determinants of salt stress tolerance. Therefore, it is quite clear that genetic manipulation of crop plants with antiporters do not bring any unique function as in the case of insect resistance or herbicide tolerance (Bt and Ht) traits.

Secondly, the Na⁺/H⁺ antiporter genes used to confer stress tolerance in transgenic plants are mostly taken from various plant species, in other words these are plants own genes. The basic salt tolerance mechanism is same in most plant species. However, differences in the levels of salt tolerance arise among different plant species due to variable activities and efficiencies of the antiporter genes. Antiporter proteins control and regulate the movement of Na⁺, K⁺ and other ions across cellular and vacuolar membranes, in order to maintain ionic homeostasis. On one hand, antiporters are not directly involved in osmolytes biosynthesis or other stress related metabolic changes. While, on the other hand, the antiporters based adjustment of ionic and osmotic homeostasis may have indirect effects on the levels of osmoregulators and other physiological determinants working in salt stress responses. It has been recently demonstrated in several studies that the vacuolar Na⁺/H⁺ antiporters play important role in cellular processes including, leaf development, fruit ripening, protein processing and trafficking, cell structure components and energy balance (Liu et al., 2010). These findings have established the fact that Na⁺/H⁺ antiporters play a diverse role in plant physiological processes other than maintaining ionic homeostasis.

Also, regarding environmental risk assessment, there are no reasons to assume that the salt tolerance-inducing Na⁺/H⁺ will have adverse effects on non-target organisms including beneficial insects, predators, parasitoids and valued soil microbe activities. There are no reports of any direct or indirect effects of either the antiporter genes and their encoding proteins nor the salt tolerance trait on insects, mammals and other organisms including soil microorganisms. The only area that needs proper attention in risk assessment of salt tolerance inducing Na⁺/H⁺ antiporter genes is to confer plants salt tolerance that may increase potential of persistence in agricultural environments and invasiveness in natural environment. Salt tolerance is a fitness enhancing trait that confers selective advantage to transgenic plants under salt stress condition. In addition, transgene escape to related non-GM plants, weedy relatives or hybrid volunteers may cause these plants to show vigorous plant growth and increase their weediness characters. In agricultural

environments, this trait may increase persistence of transgenic plants, their weedy relatives, or hybrid volunteers. The weedy relatives with increased salt tolerance, in turn, may increase burden on weed management practices in agricultural habitats. Transgene escape to weedy relatives in natural environments may also confer fitness advantage under salt stress. Weeds with salt tolerance may show enhanced plant growth and weediness characteristics resulting in spread and replacement of local plant communities. This may also affect populations of other organisms interacting with the weedy species.

FUTURE PROSPECTS, CHALLENGES AND RECOMMENDATIONS

So far, the strategy of coping salinity stress with introduction of Na^+/H^+ antiporters in transgenic plants has been proven very effective. There have been a number of transgenic plants transformed with various Na^+/H^+ antiporters that not only acquired salt stress tolerance, but also showed improvement in plant growth and seed production in some cases. Despite these achievements, there are many issues that need proper attention in order to fully exploit the potential of genetic transformation of crop plants with Na^+/H^+ antiporters. Some of these issues have been previously discussed in several reviews on transgenic plants transformed with antiporter proteins (Yamaguchi and Blumwald, 2005; Ashraf and Akram, 2009).

The ultimate goal of developing salt tolerant crop plants is to improve their yield potential under natural saline environments. So far, transgenic plants transformed with Na^+/H^+ antiporters have been evaluated for salinity stress tolerance only under control conditions. In some examples, however, transgenic plants transformed with the vacuolar antiporters and the vacuolar *AVP1* gene were studied for salt tolerance and yield improvement under greenhouse and field conditions such as in cotton (He et al. 2005; Pasapula et al., 2010). Transgenic wheat and barley with many genes including *AtAVP1* gene for improved salt and drought tolerance are under field trials for agronomic and risk assessment studies in Australia. The OGTR has issued a license to the University of Adelaide under license number (DIR 102) for limited and controlled release of up to 2340 of these transgenic lines into the environment (OGTR, 2010). In USA, Arcadia Biosciences conducted field trials on transgenic salt tolerant peanut, cotton and rice transformed with the *AtNHX1* gene.

Despite these examples, most of the transgenic plants have yet to be tested under natural stressful environments. It is highly likely that transgenic plants with high salt tolerance and improved plant growth under controlled conditions may not exhibit the same tolerance under natural stressful environments due to interaction with varying environmental factors. Under natural conditions,

severity of salt stress is likely to increase with the presence of other stresses such as changes in temperature and light intensity, water shortage, UV irradiation and soil nutrient deficiency. In addition, under natural field conditions, severity of salt stress may also increase due to the presence of other salts such as CaCl_2 , CaSO_4 and Na_2SO_4 . Moreover, in most of the experiments that were conducted on transgenic plants transformed with Na^+/H^+ antiporters, improvement in plant growth under salt stress was reported only during initial stages of plant growth such as germination and seedling growth. It is still to be explored whether the transgenic plants will maintain the same salt tolerance and improved plant growth during later stages of plant development and whether salt tolerance will be achieved on the whole plant level.

So far, the main focus of research to enhance salt tolerance of plants through Na^+/H^+ antiporter proteins has been on model plants. A few economically important crops such as wheat, rice and maize, which are salt sensitive, were also transformed with antiporter proteins but these plants did not show significantly high salt stress tolerance. Therefore, transgenic research with Na^+/H^+ antiporter proteins, isolated from various species, should be extended to these crop plants to raise their salt tolerance level at natural field conditions. In addition, many other economically important crops are sensitive to salt stress and are thus potential candidates for transformation with antiporter proteins.

Another point that also needs research focus is the use of Na^+/H^+ antiporter genes from halophytes. Thus, most of the transgenic experiments utilized Na^+/H^+ antiporters from glycophytes, mostly from *Arabidopsis*, rice and other species. Halophytes have well developed salt tolerance mechanisms and the high salt tolerance of these plants might partly be due to the high efficiency of their membrane and vacuolar Na^+/H^+ antiporter genes. Therefore research should be focused on isolation of Na^+/H^+ antiporters from various halophytes species that can survive under very high saline conditions. Introduction of these antiporters into crop plants may enhance their salt tolerance by many folds. Therefore the future transgenic development for salt tolerance should use antiporters from halophytes. In addition to this, prior to introduction of halophytic Na^+/H^+ antiporters into crop plants, the salt tolerance mechanism of both the recipient and donor plants should be well known. It is also important to know in which part of the plant, the gene is to be targeted and expressed because the recipient and donor may differ in localization and expression of the antiporter gene in different organs such as roots or shoots.

Furthermore, the monocot and dicot nature of both the recipient and the donor plant is also important. In previous transgenic experiments, the level of salt tolerance varied depending on the source of antiporters and the engineered plants. Na^+/H^+ antiporters taken from dicots and engineered in monocots did not confer high salt tolerance, and vice versa. Similar results were reported in

one study conducted by Liu et al. (2010). *Arabidopsis* was transformed with various vacuolar *NHX1s* isolated from *Arabidopsis*, cotton, *Sueda salsa*, wheat and yeast. Transgenic *Arabidopsis* plants showed salt and drought tolerance. However, the degree of salt tolerance varied depending upon the type of transgene; *GhNHX1* > *ScNHX1* > *AtNHX1* > *SsNHX1* > *TaNHX1*. The *TaNHX1* from the monocot wheat did not increase salt tolerance of the dicot *Arabidopsis*. On the contrary, Zhang et al. (2008) isolated a vacuolar Na⁺/H⁺ antiporter from the monocot halophyte species, *Aeluropus litoralis*. Expression of this *AINHX* gene in tobacco, a dicot plant conferred very high salt tolerance. The authors suggested that this halophytic gene may be an excellent choice for engineering of economically important monocot gramineous crops such as wheat and rice, which are close relatives of *A. litoralis*. In similar fashion, membrane and vacuolar Na⁺/H⁺ antiporters of other halophytes should also be focused. One such halophytic plant is *Aeluropus lagopoides*, which is a monocot and thrives well at very high saline conditions up to 1000 mM NaCl concentration (Gulzar et al., 2003). The vacuolar antiporter gene termed as *AlaNHX* was isolated from this halophytic plant and submitted to Gene bank with accession number GU199336. Such type of vacuolar Na⁺/H⁺ antiporters from halophytic plants will be very effective in conferring high salt tolerance in crop plants of the family Gramineae such as barley, maize, rice, and wheat.

Moreover, the Na⁺/H⁺ exchange activity of vacuolar antiporters are relatively low and limit their use in future molecular breeding programs. It is recommended that future research should focus on modification of the function of vacuolar antiporters through improving its Na⁺/H⁺ exchange activity. This can be done through DNA shuffling technologies. Through this technology, mutations are generated in the gene structure, which are then recombined to produce new versions of the gene. Xu et al. (2010) applied this technology on the *Arabidopsis* vacuolar *NHX1* gene and generated a new gene designated as *AtNHXS1* using yeast complementation system. Expression of this gene in yeast improved the tolerance to salt stress. They reported that *AtNHXS1* showed higher Na⁺/H⁺ exchange activity as compared to that of *AtNHX1*. Therefore, this technology has great promise for enhancing the effective use of antiporter genes in future molecular breeding for salt tolerant crop plants. In addition, engineering crop plants with multiple genes for enhancing stress tolerance and yield should be adopted. The vacuolar Na⁺/H⁺ antiporters, particularly those of halophyte species should be co-expressed with other important genes such as the vacuolar *AVP1*, cell-type specific *HKT1*, and the glycinebetaine biosynthetic *codA* gene.

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