Improving Crop Salt Tolerance: Anion and Cation Transporters as Genetic Engineering Targets

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ABSTRACT

Plant exposure to high levels of NaCl results in osmotic stress and creates ion toxicity mainly due to Cl^- and, in particular, Na^+ accumulation. In addition, salt stress impacts on nutritional homeostasis of minerals such as, Ca^{2+} and K^+. The large detrimental effects of salinity on agriculture require understanding of the underlying genes and mechanisms to improve crop tolerance. A large number of potentially important genes has been identified using forward and reverse genetics, yeast complementation and transcriptomics approaches. The present review gives an overview of membrane transporters that have been assigned functions in uptake, efflux, compartmentation and translocation of Na^+ and Cl^-. Subsequently, the review critically evaluates how specific genes were identified and assesses whether these would provide valuable targets to improve plant tolerance.

CONTENTS

INTRODUCTION

One of the most critical problems currently affecting mankind is the increasing shortage of water and land to support global food requirements. The high demand for agricultural based products must therefore, be met primarily, by increasing productivity from cultivated land as well as the use of marginal land for agricultural production (O'Leary 1995). Such endeavours are often frustrated by environmental stresses that have a large negative impact on agronomy. For example, over 800 million hectares of land are affected by high levels of salinity worldwide (FAO 2008) sharing more than 6% of the world's total land area. However, agricultural productivity in such environments can be potentially raised by growing crops with high tolerance to salt stress.

Plants can be divided into glycophytes and halophytes on the basis of their abilities to grow on different salt concentrations (Flowers *et al.* 1977). Halophytes can grow on high concentrations of salts. For example, *Atriplex vesicaria*, a species common to salt marshes, can grow and complete its life cycle at concentrations of 700 mmol L^{-1} NaCl. Although there are many facets to the remarkable tolerance of halophytes, they include effective balancing of salt accumulation to lower the tissue osmotic potential and salt compartmentation to avoid toxicity, with growth. In addition, several halophytes have specialized cells such as salt glands in leaves and stems to extrude surplus ions and thus avoid toxic levels of accumulation (Munns 2002). In contrast, the majority of terrestrial plants, including most agriculturally important crops, is glycophytic. Glycophytes typically restrict salt translocation to shoot tissues and are therefore, sometimes classified as 'excluders'. However, glycophytes appear to share most tolerance mechanisms that have been detected in halophytes but the levels and regulation of these systems are not well adapted to moderate and high levels of salt, resulting in a greater degree of sensitivity.

Invited Review

Similarly, there may be important ecophysiological variations between mono- and dicotyledonous plants: For example, in *Arabidopsis* shoot Na^+ accumulation and salt tolerance do not show a close correlation whereas this is the case for most tested cereals (Moller and Tester 2007).

There are two main components to salinity stress in plants; an initial osmotic stress and a subsequent accumulation of toxic ions which negatively affects cellular metabolism (Munns *et al.* 2006) and in addition, can lead to secondary stresses such as nutritional imbalance and oxidative stress (Alscher *et al.* 1997). The Na^+ cation is predominantly associated with the deleterious effect of salinity, and therefore, most research has focussed on this mineral. However, plant adaptation to salt stress also requires appropriate regulation of Cl^- homeostasis (Munns and Tester 2008). Indeed, for species such as soybean, citrus and grapevine where Na^+ is predominantly retained in the roots and stems, Cl^- is considered more toxic since this ion is accumulated to high levels in shoot tissues, negatively impacting on essential processes such as photosynthesis.

The osmotic component of salinity is caused by excess inorganic ions such as Na^+ and Cl^- in the environment that decrease the osmotic potential of the soil solution and hence water uptake by the plant root. Uptake of abundantly available Na^+ and Cl^- therefore, offers a comparatively cheap way to lower the tissue-osmotic potential. To avoid the risk of ion toxicity associated with this strategy, Na^+ and Cl^- are generally compartmentalized in the vacuole and/or less sensitive tissues. In parallel, adjustment of the cytoplasmic compartment is achieved via production of compatible osmolytes such as, proline, mannitol, sorbitol and glycine betaine. The latter also act as antioxidants and thus, detoxify reactive oxygen species (ROS). However, when plants are growing in high salt concentrations, an adequate sequestration of ions in the vacuole can become a limiting factor, especially in the case of glycophytes. In this scenario, plants can accumulate excessive amounts of Na^+ in the cytosol which negatively affects many aspects of cellular physiology. For example, the physicochemical similarities between Na^+ and K^+ lead to competition at transport and catalytic sites that normally bind the essential cation K^+ and maintaining a high cytosolic K^+/Na^+ ratio is believed to improve salt tolerance (Maathuis and Amtmann 1999; Zhu 2001).

Oxidative stress is another aspect of salinity stress which is in fact, a consequence of salinity-induced osmotic and/or ionic stress (Hernandez *et al.* 2001). The salt induced production of ROS such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) is counteracted by different detoxifying enzymes such as, superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2). Indeed, transgenic rice overexpressing a yeast Mn super oxide dismutase was shown to have improved salinity tolerance (Tanaka *et al.* 1999).

Plants need to sense the osmotic and ionic components of salt stress. This is important to maintain correct functioning of the roots and shoots in response to water deficit and the presence of high Na^+ concentrations. Plants show rapid responses after the addition of NaCl to the external medium (Knight *et al.* 1997) but the details are not very well understood. Increases in extracellular Na^+ are presumably sensed at the plasma membrane although no sensors have been identified as yet. A rapid increase in cytosolic free Ca^{2+} suggests that an increase in extracellular NaCl in fact, facilitates the Ca^{2+} flux into the cytosol across plasma membrane and tonoplast (Tracy *et al.* 2008). The raise in cytosolic Ca^{2+} may be relayed by Ca^{2+} sensors such as, SOS3, a calcineurin B-like protein. Although the affinity of SOS3 for Ca^{2+} is not known, increases in cytosolic Ca^{2+} likely facilitate the dimerization of SOS3 and its subsequent interaction with SOS2, a calcineurin B like interacting protein kinase (CIPK24). The SOS3/SOS2 complex is targeted to the plasma membrane through myristoylation and subsequently phosphorylates the Na^+/H^+ antiporter, SOS1 (Zhu *et al.* 2002). SOS1 has also been suggested to act as a Na^+ sensor (Shi *et al.* 2000).

Several putative osmosensors have been identified in plants. The HKT-type Na^+/K^+ co-transporters of *Eucalyptus camaldulensis* have been shown to increase ion uptake when expressed in *Xenopus laevis* oocytes exposed to hypoosmotic conditions (Liu *et al.* 2001) while the hybrid two-component histidine kinase AtHK1 activates a MAP kinase cascade in *Arabidopsis* in response to hyperosmotic conditions (Urao *et al.* 1999).

Cyclic nucleotides such as cAMP and cGMP have also been implicated in salt stress signalling. Electrophysiological data suggest that cyclic nucleotides may reduce Na^+ entry into the cell by down regulating voltage-independent cation channels in *Arabidopsis* (Maathuis and Sanders 2001) and a rapid increase in cytosolic cGMP was observed when *Arabidopsis* plants were exposed to salt and osmotic stress (Donaldson *et al.* 2004).

The large number of studies on plant salt tolerance has established that salt tolerance is a multigenic, complex trait which involves many physiological and biochemical mechanisms that vary between species with diverging resistance. However, a common theme of tolerance is the adequate control of salt uptake at the root level, regulation of influx into cells, control over long distance transport and the compartmentation at the cellular and tissue level (Blumwald 2000; Flower and Colmer 2008). These processes are mediated by membrane transporters and manipulating the activity of this class of proteins has therefore enormous potential to affect plant performance in saline conditions (Maathuis 2007). However, with around 1200-1500 transporters present in a typical plant genome (Maathuis *et al.* 2003), it remains a huge task to identify specific proteins as genuine targets for crop improvement.

Different approaches have been used to identify membrane transporters with putative functions in salt tolerance. Yeast is a widely used host for heterologous expression of plant proteins. Yeast complementation screens led to the isolation of plant transporters such as the vacuolar $Na^+:H^+$ antiporter AtNHX1 (Apse *et al.* 1999) and the plasma membrane $K^+:Na^+$ symporter TaHKT1 (Schachtman and Schroeder 1994). Loss of function mutants in the model system *Arabidopsis thaliana* helped characterise many membrane transporters including AtHKT1:1 involved in long distance Na^+ transport (Rus *et al.* 2004), the plasma membrane $Na^+:H^+$ antiporter SOS1 (Zhu 2000) and the vacuolar pyrophosphatase AtAVP1 (Gaxiola *et al.* 2001).

The majority of agriculturally important crops is glycophytic, and therefore seriously threatened by soil-salinity. It is well established that uptake, efflux, translocation and compartmentation of toxic ions (mainly Na^+ and Cl^-) provide important bases for salinity tolerance in plants, and hence, a potential avenue to improve crops. However, a lack of understanding regarding the molecular entities and complex interactions of the responsible membrane transport proteins has hindered progress in this respect. The present review focuses on the main processes that contribute to the overall homeostasis of the main ionic constituents of salinity, Na^+ and Cl^- and also analyses which specific membrane transporters have been shown, or are believed, to be involved in uptake, extrusion, long distance transport and compartmentalization of salt at the cellular and tissue level. Subsequently, the review critically evaluates the reported data to assess which proteins may be particularly suitable as engineering targets to improve crop salt tolerance.

With the genomes of various plants having been sequenced, the total complement of potential proteins involved in Na^+ and Cl^- transport can be surmised. **Fig. 1** gives an overview of the main classes of monovalent ion transporters that totals several hundreds of isoforms, often derived from large gene families. In the following sections we will analyse the potential roles of transporter classes and specific proteins regarding uptake, efflux translocation and compartmentation of salt. In addition, these sections will also evaluate which of these provide promising targets in the quest to improve crop salt tolerance.
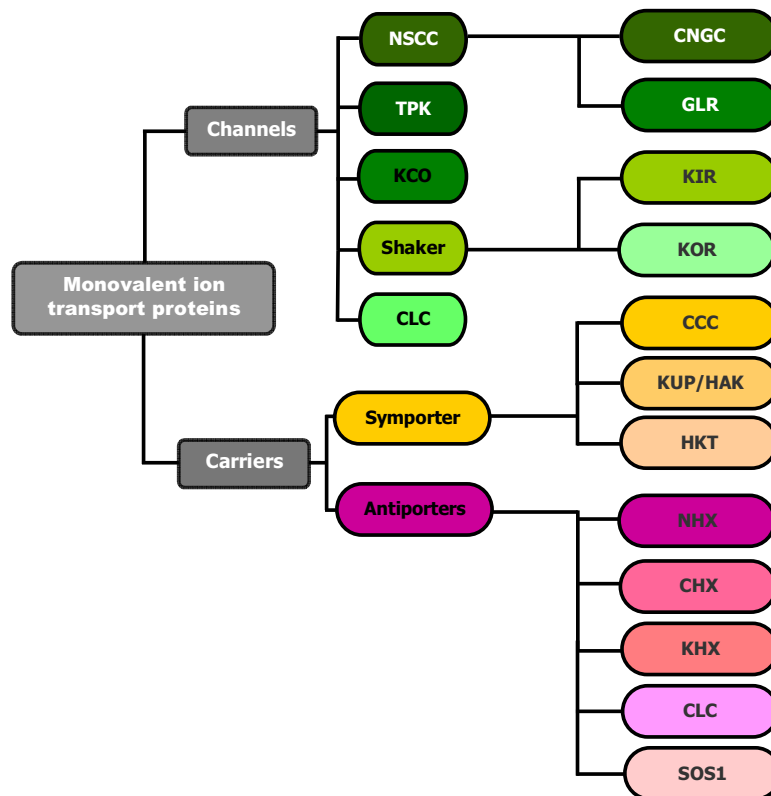
Fig. 1 Overview of main gene families involved in Na$^+$, K$^+$ and Cl$^-$ homeostasis in rice during salt stress. Abbreviations: CCC, cation chloride co-transporter; **CHX**, cation/H$^+$ exchanger; **CLC**, voltage gated Cl$^-$ channel; **CNGC**, cyclic nucleotide gated channel; **GLR**, glutamate like receptor; **HKT**, high affinity K$^+$ transporter; **KCO**, K$^+$ outward rectifying channel; **KHX**, K$^+$/H$^+$ exchanger; **KIR**, Shaker type K$^+$ inward rectifier; **KOR**, Shaker type K$^+$ outward rectifier; **KUP/HAK**, K$^+$ uptake permease; **NHX**, Na$^+$/H$^+$ exchanger; **NSCC**, non-selective cation channel; **TPK**, two-pore K$^+$ channel.

SALT UPTAKE FROM SOIL

One of the main strategies to cope with salt stress is a tight control of Na$^+$ and Cl$^-$ influx at the root:soil boundary. Whereas halophytes largely balance the uptake of inorganics with growth and osmotic requirements (Flowers and Yeo 1977), unidirectional Na$^+$ influx greatly exceeds net uptake in most glycophytes (Kronzucker *et al*. 2006). Thus, limiting influx in the latter category would potentially alleviate stress but requires detailed insights into the molecular mechanisms that underlie root Na$^+$ and Cl$^-$ uptake.

It is well established that Na$^+$ enters into root cells passively (Cheeseman 1982). Whether plants have specific transport systems for low affinity Na$^+$ uptake from soil remains an open question (Xiong and Zhu 2002) and the exact mechanisms responsible for root Na$^+$ and Cl$^-$ uptake are only partially clear and likely include transporters from several gene families and transport classes.

The role of non selective cation channels in Na$^+$ uptake

On the basis of similarity between Ca^{2+} inhibition of radio-active Na$^+$ influx and Na$^+$ current through non selective cation channels (NSCCs) it was hypothesised that the latter played a significant role in root Na$^+$ uptake (Amtmann and Sanders 1999; Tester and Davenport 2003; Maathuis 2007). However, the exact proportion and conductance of this pathway may vary substantially. For example, Kader and Lindberg (2005) employed the Na$^+$ reporter dye SBFI to directly monitor cellular Na$^+$ levels and concluded that NSCCs mediate a significantly greater proportion of overall Na$^+$ uptake in cells of salt sensitive rice cultivars.

Primary sequences indicate the presence of two major plant NSCC families, the glutamate like receptors (GLRs) and cyclic nucleotide gated channels (CNGCs), both encoded by large gene families (**Fig. 1**). In *Arabidopsis*, *CNGC3* contributes to short term Na$^+$ uptake in plant roots (**Fig. 2**) and its presence has a moderate impact on plant salt tolerance (Gobert *et al*. 2006). *AtCNGC10* was also reported to be involved in sodium uptake and long distance transport (Guo *et al*. 2008). In addition, transcriptomics studies showed significant regulation of five *CNGC* isoforms in *Arabidopsis* in response to salinity stress (https://www.genevestigator.ethz.ch/; Maathuis 2006) suggesting further members of this family may be involved.

Potassium channels may contribute to Na$^+$ uptake

Plants contain two general classes of K$^+$ selective ion channel, Shaker type channels and TPKs. Shaker type K$^+$ channels such as KAT1 and AKT1 form the predominant inward K$^+$ conductance observed in plant plasma membranes. Such channels generally have a high K$^+$:Na$^+$ selectivity and were generally regarded not to play a significant role in Na$^+$ (Schachtman *et al*. 1991; Amtmann and Sanders 1999). However, more recent work suggests that the picture is more complex and there may be ecophysiological variation in this respect. Wang *et al*. (2007) used a pharmacological approach to characterise Na$^+$ uptake in the halophyte *Suaeda maritima* and concluded that the low affinity Na$^+$ uptake pathway in this species resembles an AKT1 channel (**Fig. 2**). Similarly, Kader and Lindbergh (2005) provide evidence that K$^+$ channels mediate substantial Na$^+$ influx in a salt sensitive rice cultivar but not in a tolerant one. In both cases the conclusions were derived from applying channel blockers and inhibitors which can be notoriously non-specific but these findings do suggest K$^+$ channels are potential pathways for root Na$^+$ influx. In addition, the study by Wang *et al*. (2007) suggests that basic processes such as Na$^+$ uptake may be considerably different in halophytes and such diversity could be an important contributor to salt tolerance. However, the scarcity in data from halophytes in this respect forms a large hindrance in testing this hypothesis.
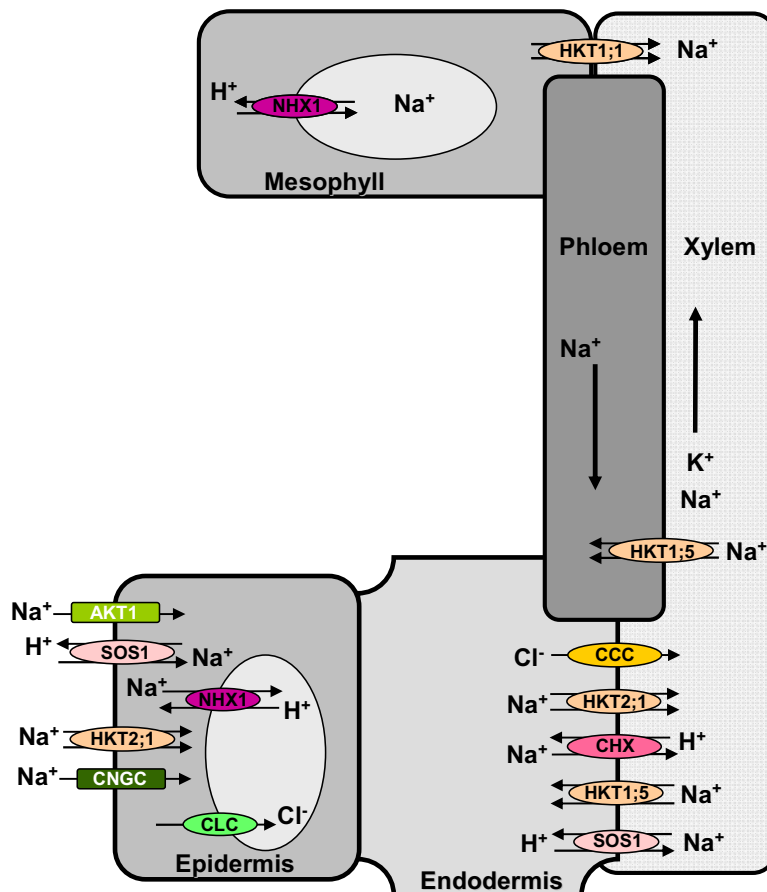
Fig. 2 Generalised functions and localisation of Na$^+$ and Cl$^-$ transporter proteins. Na$^+$ uptake at the soil-root boundary is thought to occur predominantly via non-selective cation channels like **CNGCs** and carriers from the **HKT** family. In halophytes, K$^+$ channels such as **AKT1** may also be involved in Na$^+$ uptake. AtHKT1:1 helps in controlling the accumulation of Na$^+$ in shoots and retrieval of Na$^+$ from the xylem. **HKT2;1** mediates high affinity uptake of Na$^+$ in rice but may also participate in Na$^+$ xylem loading. **HKT1;5**, located in rice xylem parenchyma cells, reduces the xylem Na$^+$ concentration and thus reduces shoot Na$^+$ load. Na$^+$ efflux into the vacuole and apoplast occurs via antiport systems such as **NHX1** at the tonoplast and SOS1 at the plasma membrane, mechanisms that are conserved across many species. **SOS1** may also mediate xylem loading of Na$^+$ along with other antiporters such as **CHXs**. Chloride uptake and transport are not well understood. Chloride channels (**CLCs**) may be involved in compartmentation of Cl$^-$ into the vacuole and chloride cation co-transporters (**CCCs**) may mediate xylem loading of Cl$^-$ in the plant. The mechanism and identity of Cl$^-$ uptake systems are not known.

Carrier type transporters that mediate Na$^+$ uptake

HKTs (high affinity potassium transporters) are carrier type proteins that mediate Na$^+$ and K$^+$ transport (Haro *et al.* 2005). HKTs were first cloned from wheat and thought to function mainly as high affinity K$^+$ uptake systems (Schachtman and Schroeder 1994) but subsequently it was found that HKTs function primarily as Na$^+$:K$^+$ symporters or Na$^+$ uniporters (Haro *et al.* 2005).

There is ample evidence that HKTs contribute to Na$^+$ uptake from the soil (**Fig. 2**). In wheat, antisense expression of wheat HKT1 showed significantly less Na$^+$ uptake in transgenic plants and enhanced growth under high salinity (Laurie *et al.* 2002). In *Arabidopsis*, only one HKT isoform is reported, i.e. *AtHKT1*, which is expressed mainly in roots and showed high levels of Na$^+$ uptake activity in both yeast and *Xenopus oocytes* (Uozumi *et al.* 2000). In *Arabidopsis*, AtHKT1 was believed to function as a potential Na$^+$ uptake pathway (Rus *et al.* 2001) but later work shows that AtHKT1 is probably mostly involved in Na$^+$ reabsorption from the shoot (Berthomieu *et al.* 2003; Sunarpi *et al.* 2005).

In contrast to *Arabidopsis*, rice contains nine *HKT* isoforms (Garciadeblás *et al.* 2003): *OsHKT 2;1* (previously *HKT1*) has been extensively studied and shown to be a high affinity Na$^+$ transporter that is of particular importance during low K$^+$ conditions (Garciadeblás *et al.* 2003; Horie *et al.* 2007). Expression of *OsHKT2;1* is localised to the root epidermis, cortical cells and vascular tissues of both roots and leaves (Golldack *et al.* 2002; Garciadeblás *et al.* 2003; Horie *et al.* 2007) and expression patterns in roots

were found to be different in salt tolerant and sensitive varieties in response to NaCl stress (Golldack *et al.* 2002). Loss of function mutants in *OsHKT2;1* show reduced growth in low K$^+$ conditions and accumulated less Na$^+$ (Horie *et al.* 2007). Thus, it appears OsHKT2;1 augments monovalent cation uptake by providing high affinity Na$^+$ uptake in K$^+$ deficient conditions. However, OsHKT2;1 relevance in Na$^+$ uptake during salinity stress may be limited since it has a micromolar affinity for Na$^+$ and its activity is rapidly down-regulated at higher ambient concentrations of Na$^+$.

OsHKT1;1 (previously HKT4) has also been studied in heterologous expression systems and found to be a low affinity Na$^+$ transporter. Transcript abundance of *OsHKT1;1* was found to be relatively insensitive when [Na$^+$] was raised from 1 to 40 mM but its transport characteristics and high expression levels in roots suggest it may form part of the low affinity Na$^+$ uptake pathway in rice (Garciadeblás *et al.* 2003).

Transcriptomics studies have also shown altered transcript levels of *OsHKT2;3* and *OsHKT2;4* in roots when plants were exposed to salt suggesting further HKTs may be involved in Na$^+$ uptake (Garciadeblás *et al.* 2003).

In addition to HKTs, other carriers have been implicated in Na$^+$ uptake. Some members of the high affinity K$^+$ uptake transporter family HAK/KUP/KT may transport Na$^+$ with low affinity in the presence of high Na$^+$:K$^+$ ratios (Pardo and Quintero 2002). Furthermore, yeast expression studies revealed that the normal function of HAK/KUP/KTs, high affinity K$^+$ uptake, is competitively inhibited by Na$^+$, pointing to a shared transport pathway of the two mono-

valent cations (Santa Maria *et al.* 1997; Fu and Luan 1998).

Several studies have shown substantial transcriptional regulation of HAK/KUP/KT isoforms by salt stress (e.g. Chao *et al.* 2005; Walia *et al.* 2005, 2007). For example, Su *et al.* (2002) observed that the expression of *HAKs* in *Mesembryanthemum crystallinum* was upregulated during salt stress and K^+-starved conditions. However, whether this result and those for other HAKs relates to a potential role in Na^+ uptake or augmentation of K^+ uptake during salinity stress remains to be established.

The low affinity cation transporter LCT1 from wheat, functions as non selective cation carrier conducting K^+, Rb^+, Na^+, and Ca^{2+} transport in yeast (Schachtman *et al.* 1997; Clemens *et al.* 1998). Expression of *LCT1* in yeast increased salt sensitivity and Na^+ contents of the cells (Amtmann *et al.* 2001) suggesting it might form a Na^+ uptake pathway in plant roots. However, the membrane localization and physiological roles of *LCT1* in plants has yet to be explored.

Transporters involved in Cl⁻ uptake

Cl^- is a major solute in plant vacuoles, particularly during salt stress, and is involved in both turgor and osmoregulation (White and Broadley 2001). In contrast to Na^+, Cl^- uptake in most conditions must be energised but although there is a substantial amount of information regarding K^+ and Na^+ transport in plants, very little is clear about the molecular mechanisms behind the substantial Cl^- influx that results from salinisation (Flowers and Colmer 2008). Plants contain CLC type anion channels which are believed to participate in turgor regulation, stomatal movement and anionic nutrient transport such as NO_3^- (Hechenberger *et al.* 1996). Although the transcript abundance of several CLCs is affected by salinity (Diédhiou and Golldack 2006), they are unlikely to contribute to root Cl^- uptake: Firstly, plant CLCs have only been detected at endomembranes which appears to exclude a role in Cl^- uptake and secondly the thermodynamics of Cl^- uptake rule out passive channel type mechanisms.

A second class of potential Cl^- transporters is formed by the cation chloride cotransporters (CCCs) encoding one gene in *Arabidopsis* and two genes in rice. AtCCC, expressed in root and shoot tissues, probably functions as a $2Cl^-:K^+:Na^+$ cotransporter. Loss of function of AtCCC in *Arabidopsis* led to a changed root:shoot Cl^- ratio but also to a large increase in net Cl^- uptake arguing against a role of AtCCC in the uptake of this ion (Colmenero-Flores *et al.* 2007). Thus, the exact mechanisms of Cl^- uptake and the involved proteins are two important questions that yet have to be answered.

TRANSPORTERS INVOLVED IN SALT EFFLUX

The previous sections showed multiple uptake pathways for salt and the apparently high membrane permeability, especially for Na^+, that has been observed in many glycophytes likely results from NSCCs and HKTs. It is therefore, essential that plants possess adequate efflux systems to remove potentially dangerous ions such as Na^+ from the cytosol. Inevitably, the mechanisms to extrude Na^+ into the apoplast or vacuole have to be energised which typically occurs via H^+ coupled antiport (Blumwald *et al.* 2000; Zhu 2001) whereas those for Cl- may be (partially) passive.

Early studies on tonoplast antiporters showed significant upregulation of their pumping capacity after plant exposure to salt (Blumwald and Poole 1985; Staal *et al.* 1991). In the plasma membrane too, evidence for $H^+:Na^+$ antiporters was obtained (Katz *et al.* 1986) underlining the relevance of such systems to plant salt tolerance.

Data are scarce for Cl- efflux: Britto and Kronzucker (2003), using compartmental flux analysis, showed large Cl^- efflux when plants were exposed to 100 mM NaCl. Just as is the case for Na^+, the majority (up to 90%) of Cl^- that entered the symplast was quickly removed. Although some

of the Cl^- efflux could theoretically be mediated by anion channels, no data are available regarding the mechanistic details or regarding the identity of the proteins involved.

Na⁺ efflux mechanisms at the plasma membrane

In *Arabidopsis*, the plasma membrane Na^+/H^+ antiporter AtSOS1 (**Fig. 2**) was identified from a forward genetics mutant screen (Zhu *et al.* 2000). *SOS1* transcription is augmented after salt stress and transport assays showed it is capable of exchanging H^+ coupled Na^+ and K^+ antiport activity (Shi *et al.* 2002). Although it may participate in other processes, the hypersensitivity displayed by loss of function mutants in SOS1 clearly show it is paramount in salt tolerance (Shi *et al.* 2003). Further work revealed that regulation of SOS1 activity by SOS3 and SOS2 also functions in rice and may be conserved in other plant species (Martinez-Atienza *et al.* 2007).

In roots, SOS1 is primarily found in root tips and around the vasculature but largely absent from other root tissue. Thus, it is unlikely that SOS1 is responsible for a large part of Na^+ efflux into the apoplast and the likelihood of additional efflux systems in epidermal and cortical cells is great.

Role of antiporters in vacuolar Na⁺ compartmentation

Na^+ compartmentation in the vacuole occurs in all tissues and is an important mechanism for osmotic adjustment and Na^+ detoxification in the cytosol. Tonoplast $H^+:Na^+$ antiport activity has been reported in many species such as sugar beet (Blumwald and Poole 1987), *Atriplex nummularia* (Hassidim *et al.* 1990), *Plantago maritima* (Staal *et al.* 1991) and *Mesembryanthemum crystallinum* (Barkla *et al.* 1995).

The primary tonoplast Na^+ antiporter appears to be NHX1 (**Fig. 2**) and has been characterised in multiple species including *Arabidopsis* (Apse *et al.* 1999), wheat (Xue *et al.* 2004), tomato (Zhang and Blumwald 2001), soybean (Li *et al.* 2006) and rice (Fukuda *et al.* 1999). Overexpression of NHX1 in several species endowed a greater level of salt tolerance. For example, transgenic tomato plants overexpressing AtNHX1 produced fruits in the presence of salt concentrations where wild type plants could not survive (Zhang and Blumwald 2001). Similarly improved salt tolerance was observed in rice after overexpression of OsNHX1 (Fukuda *et al.* 2004; Chen *et al.* 2007). However, the transgenic rice plants showed less vigorous growth in control conditions (Chen *et al.* 2007).

Other members of the NHX family are also capable of moving Na^+. Yokoi *et al.* (2002) reported that AtNHX2 and AtNHX5 could be important salt tolerance determinants, and observed that AtNHX2 has a major function in vacuolar Na^+ sequestration. In *Ipomoea nit*, the vacuolar antiporter *InNHX2* was identified which is expressed in leaves, stems and roots and its transcription is significantly induced by salt stress (Ohnishia *et al.* 2005). Heterologous expression of *InNHX2* in yeast revealed that InNHX2 catalyzes both Na^+ and K^+ transport into the vacuoles and also promotes vacuolar alkalization (Ohnishia *et al.* 2005).

Role of Cl⁻ channels in vacuolar Cl⁻ compartmentation

In addition to Na^+, Cl^- compartmentation is also important for salt tolerance, as elevated levels of Cl^- in the cytosol may be harmful, particularly in the case of citrus crops (Xu *et al.* 2000). Since the vacuole is moderately positive with reference to the cytoplasm, part of the vacuolar Cl^- sequestration could proceed through ion channels and several voltage-gated anion channels of the CLC family have been detected in the tonoplast of various species. In *Arabidopsis*, CLCa was recently shown to function primarily as a H^+ coupled antiporter to drive vacuolar nitrate accumulation

(De Angeli *et al.* 2006) whereas CLCc may also be involved in NO_3^- homeostasis rather than vacuolar Cl^- sequestration. However, CLC transcription has been found to respond to salinity: In rice, *OsCLCa* was significantly upregulated in salt sensitive cultivars in response to salinity stress and *OsCLCc*, which is expressed in both leaves and roots, showed transcript reduction in the chloride accumulating salt sensitive IR29 while transient induction occurred in the chloride excluding variety Pokkali (Diédhiou 2006). Diédhiou and Golldack (2006) showed a coordinated regulation of anion and cation homeostasis in salt-treated rice and suggested a function for *OsCLCc* in osmotic adjustment at high salinity. A similar co-regulation was recorded in soybean for *NHX1* and *CLC1* (Li *et al.* 2006). Nakamura *et al.* (2006) showed that the same CLC channels partially complimented the yeast *gef1* mutant which lacks the yeast CLC channel. In conjunction, these findings suggest that CLC type anion channels are important in mediating Cl^- sequestration in the vacuole (**Fig. 2**).

LONG DISTANCE TRANSPORT OF Na^+

Most glycophytes can be classified as salt excluders, i.e. species that prevent large accumulation of salt in photosynthesising tissues (Weicht and MacDonald 1992). Such species show a relatively high K^+:Na^+ selectivity where salt translocation is concerned, possibly via reabsorption of salts in the basal parts of the root vasculature (Lessani and Marschner 1978). In addition, retranslocation of Na^+ from shoot to root has also been described and such mechanisms would also contribute to low shoot salt loads (Pitman 1977).

In contrast, many halophytes are salt includers and store high levels of Na^+ and Cl^- in stems and leaves. Many salt includers are succulent because of the accumulation of salt in the large vacuoles of the mesophyll cells. Long distance transport of salt therefore provides another key step in the control of overall salt distribution within plant tissues (Blumwald 2000).

Several proteins have been implicated in root shoot translocation of Na^+ in plants. The plasma membrane antiporter SOS1 is expressed in root parenchyma and in Arabidopsis impacts on Na^+ loading into the xylem sap during moderate salt stress (Shi *et al.* 2002). However, its exact function may depend on the severity of the salinity stress and includes removal of Na^+ from the xylem stream when salt stress is excessive.

In *Arabidopsis*, loss-of-function mutations in the *HKT1* gene lead to overaccumulation of Na^+ in shoots and rendered the plant Na^+ hypersensitive (Mäser *et al.* 2002; Berthomieu *et al.* 2003). RNA in situ hybridizations showed that HKT1 is expressed mainly in leaf phloem tissues and mediates Na^+ loading into the phloem vessels. In addition, HKT1 may be involved in Na^+ unloading from the phloem sap in roots (Berthomieu *et al.* 2003) thus providing a mechanism for Na^+ retranslocation from shoot to root.

In other species too, HKT isoforms have been implicated in long distance Na^+ movement. In rice, OsHKT1:5 (previously HKT8) is a plasma membrane Na^+ transporter expressed in xylem parenchyma cells that retrieves Na^+ from the xylem sap (Ren *et al.* 2005). The activity of OsHKT1;5 results in less Na^+ load in shoot tissue and therefore a considerably higher K^+:Na^+ ratio in leaf tissue. OsHKT1;5 transcript abundance is responsive to salt treatment (Walia *et al.* 2005) and interestingly, shows allelic variation between cultivars that differ in salt tolerance (Ren *et al.* 2005).

In wheat, NAX1 and NAX2 were recently characterized as being involved in Na^+ exclusion in this species (Munns *et al.* 2003). Both *NAX* genes have been identified as Na^+ transporters belonging to the HKT gene family (Huang *et al.* 2006; Bryt *et al.* 2007). Like OsHKT1;5, NAX1 and NAX2 appear to remove Na^+ from the xylem whereas NAX1 is also responsible for restricting Na^+ at the leaf base.

Members of the H^+:monovalent cation exchanger family (CHX) are also likely to contribute to Na^+ translocation.

AtCHX21 is mainly expressed in the root endodermis and loss of function in this gene reduced levels of Na^+ in the xylem sap without affecting phloem Na^+ concentrations (Hall *et al.* 2006). The *Atchx21* mutant showed a complex phenotype with reduced root growth in the presence of 50 mM NaCl (Hall *et al.* 2006). In rice, salt induced expression of *OsCHX11* in roots was cultivar dependent and higher in a tolerant cultivar (Senadheera *et al.* 2009). The differential expression correlated with a higher K^+:Na^+ ratio in the tolerant cultivar suggesting that CHX11 may be involved in long distance transport of Na^+ and/or K^+.

EVALUATION OF TARGETS FOR CROP SALT TOLERANCE IMPROVEMENT

Currently, a large number of potential gene targets is available to manipulate salt tolerance. This number has drastically increased through the many large scale transcriptomics studies over the past decade but in many cases the validity of the reported findings has yet to be established.

For the various processes that contribute to salt tolerance, regulating uptake of Na^+ and Cl^- from the soil is of primary importance, particularly in glycophytes which appear to have unidirectional Na^+ and Cl^- influx that greatly exceeds net uptake. The finding that halophytes or relatively tolerant cultivars (Wang *et al.* 2007) show lower membrane Na^+ conductance agrees with the above notion. However, the details of Na^+ uptake, and even more so of Cl^- uptake, are still not clear. Although several studies convincingly show that non selective cation channels are involved their molecular nature is largely unknown. Out of the substantial gene families that encode non selective cation channels (CNGCs and GluRs) only CNGC3 and CNGC10 were shown to have a moderate impact on salt tolerance (Gobert *et al.* 2006; Guo *et al.* 2008). The data available, suggest that single CNGCs do not play important roles in Na^+ uptake. However, creating multiple loss of function mutants, e.g. for all CNGCs or GluRs expressed at the root periphery, may be required to provide more conclusive evidence in this respect.

The recent studies suggesting that inward rectifying K^+ channels may also be involved in low affinity Na^+ uptake in halophytes (Wang *et al.* 2007) and possibly rice (Kader and Lindbergh 2005) complicate the picture even further. However, for most glycophytes, this contribution is likely to be relatively small (Amtmann and Sanders 1999).

It has been well established that some members of the HKT family function as Na^+ transporter (Uozumi *et al.* 2000; Horie *et al.* 2001; Golldack *et al.* 2002) and HKTs may also contribute significantly to Na^+ uptake. In *Arabidopsis*, loss of function in AtHKT1 did not significantly affect Na^+ uptake (Berthomieu *et al.* 2003) but decreased expression of TaHKT1 in wheat did (Laurie *et al.* 2002). In rice, HKT1;1 is a likely candidate for bulk Na^+ influx during salinity (Garciadeblás *et al.* 2003), but this has yet to be confirmed. The contribution to Na^+ uptake of other carriers, e.g. of the KUP family, is likely to be small and these are therefore not of great interest in manipulating salt uptake *per se*.

Efflux and compartment of Na^+ have been shown to impact significantly on salt tolerance through modulation of transporters such as NHX1 and SOS1. The relevance of both SOS1 and NHX1 in salinity stress was originally determined in *Arabidopsis* using a forward genetic screen in the case of SOS1 (Wu *et al.* 1996) and a yeast complementation assay in the case of NHX1 (Apse *et al.* 1999). In both cases, reduction or an increase in expression correlates with reduced or increased salinity tolerance. In the case of NHX1 there is now convincing evidence that its overexpression improves tolerance in various species (Apse *et al.* 1999; Zhang *et al.* 2001; Zhang and Blumwald 2001) and it becomes clear that its transcription may be coupled to that of anion transport (Li *et al.* 2006). Thus, these antiporters not only form important targets in their own right but provide scope for synergistic interactions, for example by

improving Na^+ extrusion to the apoplast and vacuole or by combining their overexpression with increased pump activity to energise these antiporters (Gaxiola *et al.* 1999; Bartels and Sunkar 2005).

Most halophytes are 'includers' which suggests that bulk transport of Na^+ and Cl^- to shoot tissue confers some advantage. The 'excluding' behaviour of most glycophytes may point to a lack of control over this process and its manipulation may therefore be beneficial. HKTs appear to be some of the main participants in this process with both HKT1 in *Arabidopsis* and HKT1;5 in rice impacting on Na^+ translocation. Increasing evidence also implicates members of the large CHX family in this process.

CONCLUSION

Many gene targets involved in salt tolerance have been identified through various approaches, particularly through transcriptomics studies. It is likely that such approaches generate many false positives (Maathuis 2006) and this is born out by a lack of supporting evidence for an actual function in plant salinity tolerance. Caution also derives from the low level of reproducibility for identifications made through transcriptomics with most gene products appearing in only one study. Thus, it appears that forward genetics and yeast complementation strategies have so far been the most successful approaches to identify relevant targets. The accumulative data show importance of two particular classes of transporters: HKTs which function in both Na^+ uptake and long distance translocation (Ren *et al.* 2005) and NHXs in their capacity as H^+:Na^+ antiport (e.g. Apse and Blumwald 2002) or by maintaining K^+ homeostasis (Venema *et al.* 2003). The significance of these systems is often isoform dependent and may be further complicated by allelic variation between cultivars.

Manipulation of several of the genes discussed above has been shown to alter uptake, efflux, translocation and compartmentation of Na^+. Although in some of these cases improved tolerance can be observed in controlled conditions it has not yet resulted in plants with significantly improved tolerance in field conditions. A number of potential reasons can be identified for this lack of progress: field conditions are likely to vary considerably from those in controlled laboratory environments and may therefore involve different transporters. In that sense we may be 'barking up the wrong tree' and firm evidence regarding the role of transporters in natural conditions is urgently required. Also, the reductionist approach of the laboratory often neglects higher order phenomena such as interactions between fluxes of different minerals or those between various stresses. It is very likely for example that Na^+, K^+ and Cl^- fluxes are coupled and that reducing salt uptake at the root will only be successful if uptake of both Na^+ and Cl^- are inhibited.

In addition, we lack insights into the outcomes of more integrated approaches: Simultaneous upregulation of extruding mechanisms through overexpression of systems such as vacuolar pumps, NHXs and SOS1 and loss of function in uptake pathways such as non selective ion channels and HKTs promises large degrees of additive or synergistic benefits. This is technically challenging but becoming more and more feasible for example through the use of polycistronic vectors and 'gene stacking' methodology (Halpin 2005).

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