

REVIEW ARTICLE

## Na<sup>+</sup> Tolerance and Na<sup>+</sup> Transport in Higher Plants

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Tolerance to high soil [Na<sup>+</sup>] involves processes in many different parts of the plant, and is manifested in a wide range of specializations at disparate levels of organization, such as gross morphology, membrane transport, biochemistry and gene transcription. Multiple adaptations to high [Na<sup>+</sup>] operate concurrently within a particular plant, and mechanisms of tolerance show large taxonomic variation. These mechanisms can occur in all cells within the plant, or can occur in specific cell types, reflecting adaptations at two major levels of organization: those that confer tolerance to individual cells, and those that contribute to tolerance not of cells per se, but of the whole plant. Salt-tolerant cells can contribute to salt tolerance of plants; but we suggest that equally important in a wide range of conditions are processes involving the management of Na<sup>+</sup> movements within the plant. These require specific cell types in specific locations within the plant catalysing transport in a coordinated manner. For further understanding of whole plant tolerance, we require more knowledge of cell-specific transport processes and the consequences of manipulation of transporters and signalling elements in specific cell types.

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**Key words:** Review, sodium, salinity, ion transport, non-selective cation channels, long-distance transport.

### THE PROBLEMS OF SALINITY TOLERANCE

#### *Background*

High concentrations of salts in soils account for large decreases in the yield of a wide variety of crops all over the world. The problem is huge; almost 1000 million ha of land is affected by soil salinity (Szabolcs, 1994), 7 % of all land area. Of the 1.5 billion ha that is cultivated, about 5 % (77 million ha) is affected by salt (Munns *et al.*, 1999). Critically, the problem of salinization is increasing, often due to bad agricultural practices. Irrigated land is particularly at risk with approx. one-third being significantly affected by salinity. Despite its relatively small area, irrigated land is estimated to produce one-third of the world's food (Munns, 2002), so salinization of this resource is particularly critical. However, dryland salinity is also an important, and increasing, problem, at least in some areas of the world, such as in southern Australia. The recently released Environment Australia-commissioned National Land and Water Resource Audit predicts that, on a business as usual basis, 17 million ha of Australia's agricultural land will be significantly affected by salinity by 2050, this comprising 25 % of Australia's wheat belt (see <http://audit.ea.gov.au>).

The significance of soil salinity for agricultural yields is enormous. The agricultural problem of salinity tolerance is probably best tackled by either altering farming practices to prevent soil salinization occurring in the first place, or by implementing schemes to try to remediate salinized soils (such as by planting perennials to lower water tables). However, such approaches are also well complemented by

programmes to increase the salt tolerance of plants, by either traditional breeding or genetic manipulation technologies. In this way yields can be increased on affected soils whilst they are being remediated, and plants can also maintain increased growth when they encounter saline sub-soils, thus enabling them to form part of the remediation process itself.

Concentrations of various salts can be elevated in soil. For example, irrigation can lead to high concentrations of calcium and magnesium carbonates, whereas in areas with a geologically recent marine history or where there has been prolonged deposition of wind-borne marine salts, and where rainfall is too low to leach salts, soils can be high in NaCl. In addition, saline soils often have toxic concentrations of the weak acid, boric acid, whose mechanism of both transport and toxicity remain unknown.

All salts can affect plant growth, but not all inhibit growth. In addition, salts do not act alone in the soil, but interact in their effects on plants; some of these interactions are simple (e.g. interactions between Na<sup>+</sup> and Ca<sup>2+</sup>), whereas some are complex (e.g. carbonates, and their effects via increased soil pH).

Among the most common effects of soil salinity is growth inhibition by Na<sup>+</sup> and Cl<sup>-</sup>. For some plants, especially woody perennials (such as citrus and grapevines), Na<sup>+</sup> is retained in the woody roots and stems and it is the Cl<sup>-</sup> that accumulates in the shoot and is most damaging to the plant (often by inhibiting photosynthesis; Flowers, 1988). Cl<sup>-</sup> transport has been reviewed recently by White and Broadley (2001). However, for many plants (such as graminaceous crops), Na<sup>+</sup> is the primary cause of ion-specific damage. In this review, we concentrate on the effects of Na<sup>+</sup> on plants, and use the word 'salt' interchangeably with Na<sup>+</sup>.

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*Symptoms of damage*

Saline solutions impose both ionic and osmotic stresses on plants. These stresses can be distinguished at several levels. In salt-sensitive plants, shoot and to a lesser extent root growth is permanently reduced within hours of salt stress and this effect does not appear to depend on Na<sup>+</sup> concentrations in the growing tissues, but rather is a response to the osmolarity of the external solution (Munns *et al.*, 2000a; Munns, 2002). Na<sup>+</sup>-specific damage is associated with the accumulation of Na<sup>+</sup> in leaf tissues and results in necrosis of older leaves, starting at the tips and margins and working back through the leaf. Growth and yield reductions occur as a result of the shortening of the lifetime of individual leaves, thus reducing net productivity and crop yield (Munns, 1993, 2002). The timescale over which Na<sup>+</sup>-specific damage is manifested depends on the rate of accumulation of Na<sup>+</sup> in leaves, and on the effectiveness of Na<sup>+</sup> compartmentation within leaf tissues and cells. These Na<sup>+</sup>-specific effects are superimposed on the osmotic effects of NaCl and, importantly, show greater variation within species than osmotic effects (Munns, 2002).

At the molecular level, signalling mechanisms activated by salt stress include both drought-induced and Na<sup>+</sup>-specific pathways (see 'Signalling pathways' below). This review focuses on the Na<sup>+</sup>-specific effects of salinity, and particularly on the transport processes that give rise to these effects. However, it is important to bear in mind the osmotic component of salt stress, particularly when evaluating methods for screening for genotypic variation in salt sensitivity. One potentially useful method to separate ionic from osmotic components of NaCl damage is to use LiCl, a salt that is toxic at approximately one-tenth the concentration of NaCl and appears to share some signalling and transport pathways with Na<sup>+</sup> (although it may exert its toxicity via different mechanisms to Na<sup>+</sup>). However, it is unlikely that all components of osmotic and ionic stresses are completely distinct. For instance, some tonoplast Na<sup>+</sup>/H<sup>+</sup> antiporters are activated by osmotic stress, but impact on Na<sup>+</sup>-specific toxicity (see 'Pumping Na<sup>+</sup> into the vacuole' below).

Some effects of high soil Na<sup>+</sup> are also the result of deficiency of other nutrients (Silberbush and Ben-Asher, 2001), or of interactions with other environmental factors, such as drought, which exacerbate the problems of Na<sup>+</sup> toxicity. Specifically, other nutrient deficiencies can occur because elevated Na<sup>+</sup> inhibits the uptake of other nutrients by (a) disrupting the uptake of nutrients directly by interfering with transporters in the root plasma membrane, such as K<sup>+</sup>-selective ion channels; and (b) inhibiting root growth, by the osmotic effects of Na<sup>+</sup> and because of the detrimental effects of Na<sup>+</sup> on soil structure (Wild, 1988, Chapter 27). Thus, the uptake of water, growth-limiting nutrients (such as P, Fe or Zn) and the growth of soil microorganisms, such as mycorrhizal fungi, can be inhibited.

However, these effects are probably not as widespread a basis for primary Na<sup>+</sup> damage as the direct effects of Na<sup>+</sup> toxicity, and they will not be discussed further.

*The main bases for Na<sup>+</sup> toxicity*

In shoots, high concentrations of Na<sup>+</sup> can cause a range of osmotic and metabolic problems for plants. Leaves are more vulnerable than roots to Na<sup>+</sup>, simply because Na<sup>+</sup> (and Cl<sup>-</sup>) accumulate to higher concentrations in shoots than in roots. Roots tend to maintain fairly constant levels of NaCl over time, and can regulate NaCl levels by export to the soil or to the shoot. Na<sup>+</sup> is transported to shoots in the rapidly moving transpiration stream in the xylem, but can only be returned to roots via the phloem. There is limited evidence of extensive recirculation of shoot Na<sup>+</sup> to roots, suggesting that Na<sup>+</sup> transport is largely unidirectional and results in progressive accumulation of Na<sup>+</sup> as leaves age.

Metabolic toxicity of Na<sup>+</sup> is largely a result of its ability to compete with K<sup>+</sup> for binding sites essential for cellular function. More than 50 enzymes are activated by K<sup>+</sup>, and Na<sup>+</sup> cannot substitute in this role (Bhandal and Malik, 1988). Thus, high levels of Na<sup>+</sup>, or high Na<sup>+</sup> : K<sup>+</sup> ratios can disrupt various enzymatic processes in the cytoplasm. Moreover, protein synthesis requires high concentrations of K<sup>+</sup>, owing to the K<sup>+</sup> requirement for the binding of tRNA to ribosomes (Blaha *et al.*, 2000) and probably other aspects of ribosome function (Wyn Jones *et al.*, 1979). The disruption of protein synthesis by elevated concentrations of Na<sup>+</sup> appears to be an important cause of damage by Na<sup>+</sup>.

Osmotic damage (i.e. osmotically driven removal of water from cells) could occur as a result of the build up of high concentrations (possibly several hundred mM) of Na<sup>+</sup> in the leaf apoplast, since Na<sup>+</sup> enters leaves in the xylem stream and is left behind as water evaporates. This mechanism of Na<sup>+</sup> toxicity was first proposed by Öertli (1968), and direct supporting evidence has been provided by x-ray microanalysis measurements of Na<sup>+</sup> concentrations in the apoplast of rice leaves by Flowers *et al.* (1991). These authors calculated that there was about 600 mM Na<sup>+</sup> in the apoplast of leaves of rice plants that were moderately salt-stressed. The universal applicability of the importance of apoplastic Na<sup>+</sup> accumulation is nevertheless in question. By infiltrating leaves with water and rapidly washing it out by centrifugation, Muhling and Läuchli (2002) failed to measure large concentrations of Na<sup>+</sup> in the leaf apoplast of salt-stressed maize and cotton. How much these contrasting results reflect biological or technical differences remains to be seen.

The cellular toxicity of Na<sup>+</sup> causes another type of osmotic problem. Plants need to maintain internal water potential below that of the soil to maintain turgor and water uptake for growth. This requires an increase in osmoticity, either by uptake of soil solutes or by synthesis of metabolically benign ('compatible') solutes. This drought component of salinity poses a dilemma for plants: the major, cheap solutes in saline soils are Na<sup>+</sup> and Cl<sup>-</sup>, but these are toxic in the cytosol (see below). Compatible solutes are non-toxic, but are energetically much more expensive.

With high concentrations of Na<sup>+</sup> in the leaf apoplast and/or vacuole, plant cells have difficulty maintaining low cytosolic Na<sup>+</sup> and, perhaps as importantly, low Na<sup>+</sup> : K<sup>+</sup> ratios (Gorham *et al.*, 1990; Dubcovsky *et al.*, 1996; Maathuis and Amtmann, 1999). Cytosolic Na<sup>+</sup>

concentrations are probably in the order of 10–30 mM [measured in plants grown at various NaCl concentrations ranging from 1–200 mM using x-ray microanalysis [Jeschke and Stelzer, 1976; Behl, cited in Jeschke (1984); Koyro and Stelzer, 1988] or ion-sensitive microelectrodes (Carden, 1999)], although there have been reports of concentrations of 100 mM or even higher in saline conditions (Harvey, 1985; Hajibagheri *et al.*, 1985, 1987; see also Cheeseman, 1988).

#### *Methods by which to study the bases for Na<sup>+</sup> tolerance*

Physiological studies of salinity tolerance have undoubtedly benefited from advances in molecular and cellular techniques, but many experiments could be improved by improvements in the method of plant growth and manipulation. For example, measurements of responses over short times after a rapid and large change in salinity are inappropriate for the study of responses relevant to plant growth in saline soils where smaller, slower changes occur.

In addition, if Na<sup>+</sup>-specific toxicity is the subject of study, then care must be taken to distinguish those effects due to the osmotic component of salinity. For instance, plants growing in conditions of very low transpiration, such as on a sterile plate, are likely to be more sensitive to the osmotic component of salinity (as Na<sup>+</sup> delivery to the shoot will be reduced). Likewise, inhibition of root elongation is probably not a good measure of Na<sup>+</sup>-specific toxicity, because it is not well correlated with Na<sup>+</sup> transport to the shoot (Munns, 2002, but see the discussion of *sos* mutants below).

The choice of model systems to be used also requires more careful consideration. For example, results from our laboratory suggest that tobacco appears to be more sensitive to the low osmotic potential of growth solutions containing NaCl, rather than to the specific effects of Na<sup>+</sup> (or Cl<sup>-</sup>) (Murthy and Tester, 1996). Thus, some studies in these plants using addition of NaCl may be more relevant to studies of drought tolerance rather than to salinity tolerance in particular.

We have also found that there is no relationship between Na<sup>+</sup> accumulation and salt sensitivity in *Arabidopsis thaliana*. Likewise, as detailed later in this review, mutations that increased salt sensitivity of arabidopsis often did not cause increased accumulation of Na<sup>+</sup> (see, for example, Mechanisms of salinity tolerance, Introduction). Moreover, although addition of Ca<sup>2+</sup> reduced Na<sup>+</sup> influx and accumulation in arabidopsis, it did not alleviate salt-induced inhibition of growth (P. A. Essah, R. Davenport and M. Tester, unpubl. res.). Although our observations are similar to reports of the Ca<sup>2+</sup>-insensitivity of some brassicas (Ashraf and Naqvi, 1992; Schmidt *et al.*, 1993), they contrast with results for many crop species, where Na<sup>+</sup> accumulation is correlated with toxicity, and supplemental Ca<sup>2+</sup> both reduces Na<sup>+</sup> accumulation and improves growth in saline conditions (Rengel, 1992; Cramer, 2002). This does not preclude the use of these model plants for the study of Na<sup>+</sup> transport, but their use as a model for studying salinity tolerance in crop plants may be limited. In addition to highlighting the need for caution when using model plants, these considerations also recognize the diversity of plant

responses to salinity, and the difficulty in making generalizations about plant responses to elevated salinity. These issues are addressed further below.

In addition to comparative physiological studies, techniques exploiting the ability to manipulate levels of expression of genes are starting to provide insights into the mechanisms of salinity tolerance. Both misexpression of targeted genes (e.g. Zhang and Blumwald, 2001) and the random alteration of genes (e.g. Zhu, 2000) have been used to good effect. One feature of note with random mutagenesis studies, though, is that many more salt-sensitive than salt-tolerant mutants appear to have been identified {Tsugane *et al.* (1999) and Quesada *et al.* (2000) being notable published exceptions for arabidopsis; and Zhang *et al.* (1999) for rice [The salt tolerant mutants identified by Saleki *et al.* (1993) and Werner and Finkelstein (1995) are not included here, because they were mutants tolerant only in germination, and this tolerance did not appear to extend to the growth of seedlings.]}. It is not known if this is related to the fact that most screens appear to use knock-out mutagenesis rather than activation mutagenesis, or to the fact that the mutants studied to date have had activity affected constitutively rather than in specific cell types.

This difference in numbers of tolerant and sensitive mutants also begs the question of whether tolerance is necessarily the opposite of sensitivity, or if salt-sensitive mutants are revealing processes distinct from those involved in tolerance. Another unusual feature of the mutagenesis studies published to date is that genes shown to affect salinity tolerance encode proteins involved in cytosolic or transport processes—we know of no transcription factors that have yet been identified from mutant screens. This is in stark contrast to screens for developmental mutants, for example. Given the potentially complex (multigenic) nature of salinity tolerance, forward genetic screens are an appropriate strategy for revealing genes involved in these processes, which makes the lack of appearance of genes controlling cellular processes at a higher level (i.e. transcription factors) the more surprising.

## MECHANISMS OF SALINITY TOLERANCE

### *Introduction*

We know that some plants tolerate high soil salinity better than others; agriculturally, yield is not reduced as much at a given salinity for some plants. This variability occurs between major groups of plants (mangroves and chenopods are examples of taxonomically distinct groups of plants dominated by salt-tolerant species), between closely related species, and between different varieties or even individuals within a varietal line. Differences between closely related plants are particularly interesting because they suggest that it may be possible to identify a small number of factors that influence salt tolerance.

Differences in yield under saline conditions often simply reflect differences in plant vigour. For example, Quarrie and Mahmood (1993) found a very close correlation between yield in control conditions and that in salinized conditions (Fig. 1). Likewise, vernalization can confer a degree of

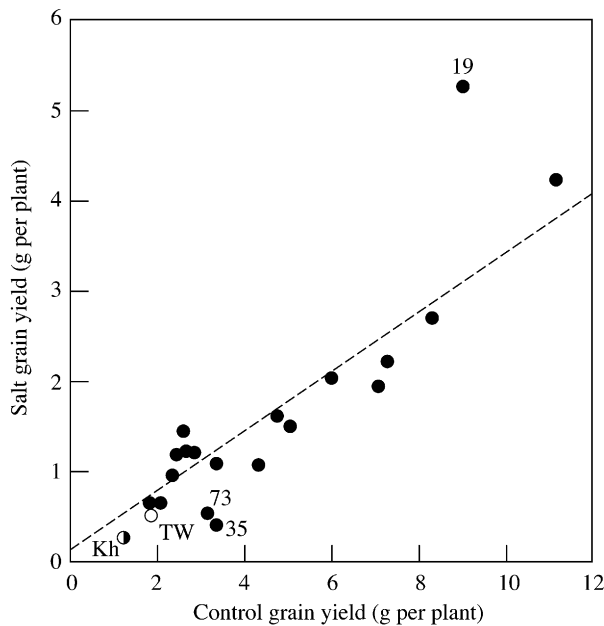


FIG. 1. Correlation between wheat grain yields in control and salinized conditions; from Quarrie and Mahmood (1993). Line 19 shows unusually high growth in salinized conditions relative to its performance in control conditions.

salinity tolerance (Taeb *et al.*, 1992), as can earlier development. In fact, breeding for more vigorous plants has been argued to be agronomically the most effective approach for increasing yield in saline soils (Richards, 1992). However, such an approach must have its limits, with yield increases as a result of increased vigour now reaching their limit (Conway, 1997), and a strategy whereby plants are selected for an ability to maintain yields in salinized conditions must also be employed. If extra tolerance can be conferred on top of other desirable features, then this must surely be beneficial.

By selecting Triticum genotypes with similar biomass and growth rates under control conditions, Schachtman and Munns (1992) demonstrated that variations in leaf growth under saline conditions were correlated with leaf concentrations of  $\text{Na}^+$ , independent of plant vigour. This type of variation is also evident in Fig. 1: line 19 showed unusually high productivity in highly saline conditions, and this was correlated with unusually low  $\text{Na}^+$  concentrations in the shoot. Empirically, for a wide range of species, it is often found that plants that are more able to tolerate moderately saline environments have a greater ability to exclude  $\text{Na}^+$  from the shoot, or at least the leaf blades, and concurrently maintain high levels of  $\text{K}^+$  (e.g. Greenway, 1962; Gorham, 1990; Schachtman *et al.*, 1992; Colmer *et al.*, 1995; Dubcovsky *et al.*, 1996; Munns *et al.*, 2000b; Flowers and Hajibagheri, 2001; Zhu *et al.*, 2001). This has been found in crop plants bred for salinity tolerance, as well as in crops that were screened for tolerance (having been selected originally for other properties), and in wild relatives of some crop plants (e.g. Nevo *et al.*, 1992).

This correlation appears to be particularly strong in the graminaceous crop species, although a notable exception to this generalization appears to be maize (Alberico and Cramer, 1993; Cramer *et al.*, 1994; N. Al-Mansour and M. Tester, unpubl. res.), with growth inhibition being correlated with sensitivity of leaf extension to salt-stimulated increases in abscisic acid (ABA) (Cramer and Quarrie, 2002). Another exception is rice. Although sensitivity of rice cultivars to  $\text{Na}^+$  is related to shoot  $\text{Na}^+$  accumulation, the transport characteristics of  $\text{Na}^+$  in rice have been shown by Flowers and colleagues to be quite distinct from those of other cereals, with a large component of  $\text{Na}^+$  influx into roots being due to 'leakage' past the endodermis (Yeo *et al.*, 1987, 1999; Yadav *et al.*, 1996) (see 'Bypass flow', below).

Dicotyledonous species vary more than monocotyledonous species in the extent to which tolerance is associated with low shoot  $\text{Na}^+$ . For example, a highly salt-tolerant wild relative of tomato, *Lycopersicon peruvianum*, accumulates higher concentrations of  $\text{Na}^+$  than the salt-sensitive domesticated tomato, *L. esculentum* (Tal, 1971; Santa-Cruz *et al.*, 1999). Importantly, the sensitivity of wild-type arabidopsis (P. A. Essah, R. Davenport and M. Tester, unpubl. res.), and that of salt-sensitive (Zhu *et al.*, 1998) and salt-accumulating (Nublat *et al.*, 2001) mutants does not appear to be closely related to shoot levels of  $\text{Na}^+$ . For example, the salt-sensitive mutant of arabidopsis, *sos1*, has a lower shoot  $\text{Na}^+$  content and lower  $\text{Na}^+$  influx than wild-type plants (at least in the presence of low  $\text{K}^+$ ) (Ding and Zhu, 1997).

This relationship between salt tolerance and low shoot  $\text{Na}^+$  does not hold for halophytes (for present purposes considered as those plants that show little growth reduction at  $\text{NaCl}$  concentrations of 300 mM or more). Some halophytes can accumulate extravagant quantities of  $\text{Na}^+$  in the shoot (up to 50 % of dry weight) without dying. The tendency to accumulate high concentrations of  $\text{NaCl}$  in the shoot is particularly associated with dicotyledonous halophytes, and in these plants  $\text{NaCl}$  may account for almost the total osmotic potential of the shoot (that is,  $\text{NaCl}$  is employed preferentially as an osmoticum; Flowers and Yeo, 1986; Glenn *et al.*, 1999). Monocotyledonous halophytes tend instead to take up less  $\text{Na}^+$  and maintain higher  $\text{K}^+$  in the shoot than dicotyledonous halophytes, and osmotic balance is achieved partially by sugar synthesis. Interestingly, there seems to be large variation even within halophytic taxa with respect to strategies. For example, chenopods vary in the extent of  $\text{Na}^+$  uptake and  $\text{K}^+ : \text{Na}^+$  ratios in the shoot (Reimann and Breckle, 1993), and mangroves may employ different strategies depending on whether or not they have salt glands (Atkinson *et al.*, 1967).

Although dicotyledonous halophytes are often considered to 'accumulate'  $\text{Na}^+$  in the shoot, it is unlikely that halophytic species have higher rates of  $\text{Na}^+$  transport at high salinities than salt-sensitive plants. In fact, halophytes are much more effective at controlling uptake of  $\text{Na}^+$  than salt-sensitive plants (see 'Pathways for initial entry into the root'). It is probably only the fact that they live longer than glycophytes at higher salinities that leads to the high concentrations of shoot  $\text{Na}^+$  often observed. Studies of halophyte ion transport at low salinities tend to obscure this, because many halophytes show a growth stimulation upon

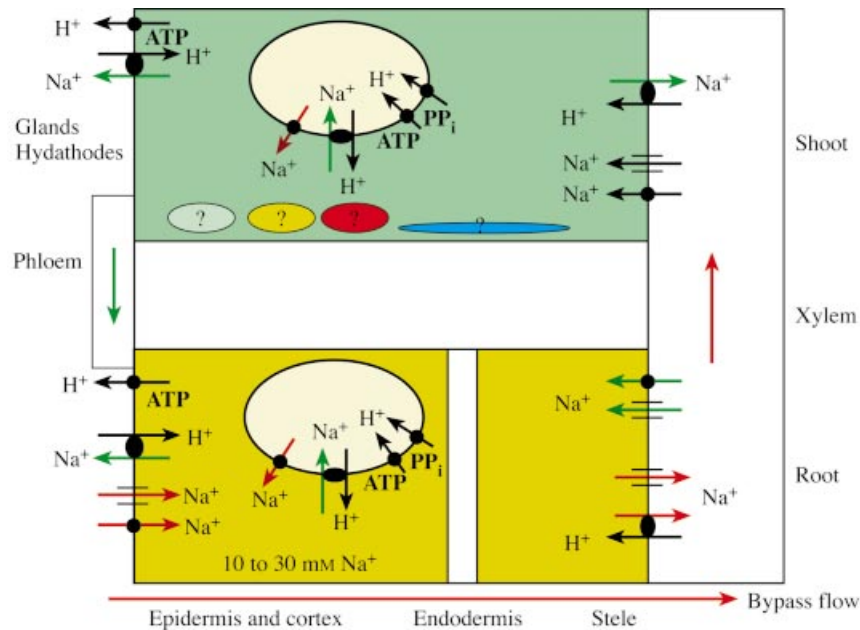


FIG. 2.  $\text{Na}^+$  transport processes influencing  $\text{Na}^+$  tolerance in higher plants. Red arrows indicate  $\text{Na}^+$  movement, the minimization of which would increase tolerance; green arrows represent  $\text{Na}^+$  movements, the maximization of which would increase tolerance. The coloured shapes in the leaf represent chloroplasts (green), mitochondria (orange), peroxisomes (red) and endoplasmic reticulum (dark blue).  $\text{Na}^+$  transport processes into and out of these organelles is unknown. Vacuoles are represented by light blue shapes.

addition of  $\text{NaCl}$  to the growth medium due to accumulation of  $\text{NaCl}$  at moderate salinities (the growth optimum varies for different species, and may be as high as 400 mM  $\text{NaCl}$  or more).

Although  $\text{NaCl}$  accumulation in these halophytes are often higher than those in glycophytes at low salinities, uptake is not proportional to external salinity, and is curtailed at higher salinities (e.g. Glenn *et al.*, 1999; Munns *et al.*, 1999). By comparing external and xylem  $\text{Na}^+$  concentrations, Munns *et al.* (1999) estimated that at 200 mM external  $\text{Na}^+$ , about 97 % of all  $\text{Na}^+$  presented to the root surface must be excluded—whether in a glycophyte or halophyte. Moreover, where it has been examined, it has been found that  $\text{Na}^+$  concentrations in halophytes are lower in growing tissues than in mature ones, implying a need, in common with glycophytes, to protect metabolically active cells from  $\text{Na}^+$  (Flowers and Yeo, 1986). This issue is discussed further below, but here we can say that evidence from studies of halophytes indicates that the ability to regulate  $\text{Na}^+$  uptake and transport to the shoot is critical in all plants. This seems to hold true even for halophytes able to secrete  $\text{Na}^+$  from the shoot. For instance, in two mangrove species,  $\text{Na}^+$  excretion via salt glands does not keep pace with  $\text{Na}^+$  uptake over a range of salinities, reducing plant growth as salinity increases (Ball, 1988).

Although halophytes probably do not accumulate salt any faster than glycophytes, they nevertheless end up with shoot concentrations of  $\text{Na}^+$  that are lethal to glycophytes. Thus, control of  $\text{Na}^+$  transport must operate at several levels:  $\text{Na}^+$  entry and supply to the shoot must be controlled, and the  $\text{Na}^+$  that reaches the shoot (and the interior of root cells) must be partitioned to avoid deleterious osmotic and cytotoxic effects. While there is evidence that some

halophytes may have slightly modified forms of some cytosolic enzymes (Flowers and Dalmond, 1992), it is generally considered that high shoot concentrations of  $\text{Na}^+$  in halophytes imply compartmentation into the vacuole. The broad differences between halophytic monocotyledons and dicotyledons have been attributed to differences in water content and hence vacuolar volume. Dicotyledons may maintain higher shoot  $\text{Na}^+$  and  $\text{Na}^+ : \text{K}^+$  ratios because they can store most of the  $\text{Na}^+$  in the vacuole and require relatively little  $\text{K}^+$  for cytosolic metabolism, whereas monocotyledons may have less  $\text{Na}^+$  storage capacity, and require more  $\text{K}^+$  and compatible osmotica for cytosolic compartments (Flowers and Yeo, 1988; Glenn *et al.*, 1999).

In this review, we shall restrict ourselves to considerations of plant adaptations to moderate levels of salinity, which are those most likely to be of agricultural relevance. However, we will argue, where appropriate, that halophytes do not necessarily differ in kind from salt-sensitive species, but in general are more effective in employment of common mechanisms to avoid salt damage. Mechanisms to minimize damage from high salinity vary among plants, and several mechanisms must operate in a coordinated fashion to manage  $\text{Na}^+$  (Fig. 2). For example, plants can: (1) minimize initial entry; (2) maximize efflux; (3) minimize loading to the xylem or maximize retrieval before reaching the shoot; (4) maximize recirculation out of the shoot in the phloem; (5) maximize intracellular compartmentation or allocation to particular parts of the shoot (e.g. pith cells or old leaves); or even (6) secrete salt onto the surface of the leaf.

In this review, we shall separate adaptations involving processes common to all cells within the plant ('cellular adaptations') from those involving processes specific to particular cell types within the plant ('whole plant adapta-

tions'). We have organized this review in such a way because we consider that there are two quite distinct mechanisms of  $\text{Na}^+$  tolerance: (1) the tolerance of single cells to high salinity, involving, for example, intracellular compartmentation and damage repair; and (2) tolerance at a higher level than that of the single cell, involving, for example, control of long-distance transport and the site of  $\text{Na}^+$  accumulation within the plant. A review by Neumann (1997) also discusses these issues, and this division is also employed by Adams *et al.* (1992) when comparing responses of *Mesembryanthemum* tissue culture and intact plants. Both levels of process are clearly important, and for tolerance of a whole plant, the relative importance of these two mechanisms probably varies with species and conditions.

The need for multiple responses to salinity within a plant is indicated by the high rate of failure of programmes to regenerate stably salt-tolerant plants from salt-tolerant cells selected in tissue culture systems (Dracup, 1991; Winicov, 1998). Thus, salt-tolerant cells rarely regenerate to produce fertile salt-tolerant plants with heritable salt tolerance, consistent with cell-specific processes usually being more important than general cellular processes. The reverse finding is also pertinent, namely that calli derived from naturally occurring salt-tolerant plants do not reflect the tolerance of the plants from which they were derived (Smith and McComb, 1981).

Recent genetic manipulations showing increased tolerance resulting from constitutive overexpression of specific genes (e.g. Apse *et al.*, 1999; Sugino *et al.*, 1999; Gaxiola *et al.*, 2001; Piao *et al.*, 2001; Rus *et al.*, 2001a; Zhang and Blumwald, 2001; Zhang *et al.*, 2001; Pandey *et al.*, 2002) raises questions about the general validity of this thesis. Although it is not known whether such manipulations do, in fact, generate salt-tolerant cells per se, they are altering expression in all cell types within a plant, indicating that manipulation of several processes at the cellular level can increase salinity tolerance of plants. It is interesting that such manipulations appear not to have been selected for when salt-tolerant cells were selected in tissue culture systems.

### Cellular adaptations to high $\text{Na}^+$

#### 1. Intracellular compartmentation—with synthesis of 'osmoprotectants'

The most direct way to maintain low cytoplasmic  $\text{Na}^+$  is to sequester it in vacuoles within each plant cell. To maintain equal osmotic potentials in vacuole and cytoplasm, solutes whose accumulation is not detrimental to cellular biochemistry must accumulate in the cytoplasm. Intracellular compartmentation can also be associated with succulence, to provide a larger volume of vacuole in which the  $\text{Na}^+$  can be stored.

*Pumping  $\text{Na}^+$  into the vacuole.* In the process of intracellular compartmentation,  $\text{Na}^+$  enters leaf cells and is then pumped into the vacuole before concentrations increase in the cytoplasm. The pumping of  $\text{Na}^+$  into the

vacuole is catalysed by a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter, the difference in  $\text{H}^+$  being initially established by  $\text{H}^+$ -pumping ATPase and pyrophosphatase proteins (Blumwald *et al.*, 2000).  $\text{Na}^+/\text{H}^+$  antiporter activity can increase upon addition of  $\text{Na}^+$  (e.g. in barley roots: Gabarino and DuPont, 1989; in tomato roots: Wilson and Shannon, 1995; in sunflower roots: Ballesteros *et al.*, 1997), and this induction was found to be much greater in the salt-tolerant species, *Plantago maritima*, than in the salt-sensitive species, *P. media* (Staal *et al.*, 1991). Consistently, salinity did not induce tonoplast  $\text{Na}^+/\text{H}^+$  antiport activity in salt-sensitive rice (Fukuda *et al.*, 1998). This induction is reflected in increased transcript levels of some members of the arabidopsis *AtNHX* gene family which encode vacuolar  $\text{Na}^+/\text{H}^+$  antiporters (Yokoi *et al.*, 2002). Salinization also induces activity of both vacuolar primary  $\text{H}^+$  pumps, although this appears to occur in both  $\text{Na}^+$ -tolerant and  $\text{Na}^+$ -sensitive species (reviewed by Hasegawa *et al.*, 2000; Maeshima, 2000).

The central importance of vacuolar sequestration has recently been underlined by experiments in which constitutive overexpression of vacuolar transporters has greatly increased salinity tolerance of a range of species. Overexpression of an arabidopsis vacuolar  $\text{Na}^+/\text{H}^+$  antiporter (*NHX1*) increased salinity tolerance of arabidopsis (Apse *et al.*, 1999), tomato (Zhang and Blumwald, 2001) and *Brassica napus* (Zhang *et al.*, 2001), and overexpression of the native vacuolar  $\text{H}^+$ -translocating pyrophosphatase gene (*AVPI*) increased salinity tolerance of arabidopsis (Gaxiola *et al.*, 2001). The apparent  $\text{K}^+$ -transporting activity of the arabidopsis  $\text{Na}^+/\text{H}^+$  antiporter (Zhang and Blumwald, 2001; Venema *et al.*, 2002) does not appear to cause perturbations in cytosolic  $\text{K}^+$  homeostasis, at least under the experimental conditions tested to date. Likewise, overexpression does not appear to have interfered with the possible roles for the tonoplast  $\text{Na}^+/\text{H}^+$  antiporter in control of pH of the cytoplasm (Viehveger *et al.*, 2002) or vacuole (Fukada-Tanaka *et al.*, 2000).

The mechanisms underlying the enhancement of salinity tolerance by these transporters are not yet clear. Although overexpression of *NHX1* in arabidopsis caused a small increase in shoot  $\text{Na}^+$  accumulation (Apse *et al.*, 1999), further experiments at lower salinities, where control plants are still alive, are required to confirm this for tomato and *B. napus*. While increased uptake of  $\text{Na}^+$  to shoot vacuoles could facilitate osmotic adjustment (as for halophytes), enhanced activity of  $\text{Na}^+$  compartmentation mechanisms would also confer greater tolerance by reducing  $\text{Na}^+$  accumulation in the cytosol, without any change in cellular  $\text{Na}^+$  concentrations. This is possible because  $\text{Na}^+$  transport across the tonoplast is bidirectional and dynamic: a 'leak' of  $\text{Na}^+$  from the vacuole to the cytosol (discussed below) would require compensatory pump activity to maintain constant levels of cytosolic  $\text{Na}^+$ . A higher level of tonoplast active  $\text{Na}^+$  transport may allow transgenic plants to exclude  $\text{Na}^+$  more effectively from the cytosol. It has also been suggested that overexpression of *NHX1* could cause 'spill-over' of the protein from the tonoplast to the plasma membrane pathway, resulting in enhanced cellular  $\text{Na}^+$  exclusion (Frommer *et al.*, 1999). In the case of plants overexpressing the vacuolar pyrophosphatase gene *AVPI*,

there was both increased accumulation of Na<sup>+</sup> and enhanced tolerance compared with wild-type plants (Gaxiola *et al.*, 2001), suggesting a significant role for vacuolar sequestration. Given the higher levels of Na<sup>+</sup> measured in shoots of transgenic arabidopsis plants, these experiments suggest that it is not increased exclusion from the shoot per se that is important, at least in arabidopsis, but the ability to compartmentalize effectively the Na<sup>+</sup> that reaches the shoot.

These results have several implications. First, it is remarkable that the overexpression of a single native gene product can produce such striking increases in salt tolerance. In arabidopsis, *NHX1* and a number of related *NHX*-type genes are ubiquitously transcribed, and show up-regulation by drought and/or NaCl treatments (Yokoi *et al.*, 2002). That a further constitutive increase in expression can cause an increase in salt tolerance begs the question of why breeding programmes have failed to select for such a radical increase in tolerance. It is possible that such a manipulation comes with a significant cost in yield, and that this has yet to be revealed in the glasshouse-based trials that have been undertaken to date.

Another implication of these results is that up-regulation of a component of tonoplast transport appears to have caused pleiotropic up-regulation of other genes (or of the activity of the gene products) to enable appropriate levels of Na<sup>+</sup> accumulation to occur. For example, for overexpression of H<sup>+</sup>-translocating pyrophosphatase activity to cause greater accumulation of Na<sup>+</sup> in the vacuoles (Gaxiola *et al.*, 2001), increased vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter activity must have occurred, as well as increased synthesis of osmoprotectants (see below). Elucidating the mechanism(s) for such up-regulation is an area of much interest—we do not even know at present if increased transcription or translation of the necessary genes is occurring, or whether all up-regulation is occurring at the post-translational level. In this context, it is noteworthy that constitutive overexpression of a protein kinase also increased both tolerance and Na<sup>+</sup> accumulation in arabidopsis (Piao *et al.*, 2001).

Another interesting output from these experiments is that plants are accumulating high levels of Na<sup>+</sup> whilst retaining the ability to maintain high rates of growth. Thus, extraordinarily, such plants could prove able to reduce significantly the equilibrium concentrations of Na<sup>+</sup> in saline soils. For example, given a dry matter production of 10 tonnes per hectare and a tissue Na<sup>+</sup> concentration of 5 %, 0.5 tonnes per hectare of Na<sup>+</sup> could be removed in above-ground biomass. This is equivalent to 10 % of the amount of Na<sup>+</sup> per hectare in 1 m depth of soil containing 10 % water (on a volume basis) at a concentration of just over 200 mM Na<sup>+</sup>. If these plants can sustain high rates of growth whilst accumulating such high levels of Na<sup>+</sup> in field situations, this does provide possibilities for a degree of phytoremediation of salinized lands. However, removal and disposal of the above-ground biomass would be expensive relative to the value of much salinized land, so this is probably a practical option only for high-value lands (and in cases where soil salt levels are not continuing to increase owing to aerial inputs, irrigation or rising saline water tables).

*Na<sup>+</sup> leaking back out of the vacuole.* In parallel with the pumping into vacuoles of Na<sup>+</sup> is a leak back into the cytoplasm, probably through ion channels. Cation-selective channels, such as the so-called 'SV' and 'FV' channels, are particularly abundant in the vacuolar membrane of plant cells. Based on their selectivity, these channels are classified as non-selective cation channels (Demidchik *et al.*, 2002), and are generally thought to be highly permeable to Na<sup>+</sup> as well as other cations, such as K<sup>+</sup>. If open in intact cells, this could readily cause problems for plant cells, compromising their ability to sequester Na<sup>+</sup> in the vacuole. However, because of the large number of interacting controls on the activity of these channels, their activity *in vivo* is still uncertain (Demidchik *et al.*, 2002). Nevertheless, given their relatively high permeability to cytotoxic cations (such as Na<sup>+</sup> and Ca<sup>2+</sup>), it must be presumed that their activity is low.

This presumption is supported by the observation that there are no major differences in the properties of vacuolar channels in salt-tolerant and salt-sensitive species of *Plantago* (Maathuis and Prins, 1990). Likewise, there were no particular features of the vacuolar membrane from leaves of the extreme halophyte, *Suaeda maritima* (Maathuis *et al.*, 1992). One interesting observation, however, is that plants of *Plantago* that had been grown at high NaCl concentration had much reduced channel activity in their vacuolar membrane compared with those grown at low NaCl concentration (Maathuis and Prins, 1990), implying that the energetic cost of recycling leaked Na<sup>+</sup> back to the vacuole may be significant.

To summarize, we know very little about the control of Na<sup>+</sup> efflux from the vacuole, and thus know little about the possibilities for manipulation of the control of this pathway to increase the ability of cells to sequester Na<sup>+</sup>.

*Synthesis of osmoprotectants.* As Na<sup>+</sup> accumulates in the vacuole, osmotic potential in the cytoplasm must be balanced with that in the vacuole. This is done by the synthesis and accumulation in the cytoplasm of organic solutes that do not inhibit biochemical reactions. In fact, many of them appear to protect such reactions from increased levels of inorganic ions (e.g. Shomer-Ilan *et al.*, 1991). Such 'osmoprotectants' (or 'compatible solutes') are often highly soluble, neutral or zwitterionic compounds and include secondary metabolites such as quaternary ammonium compounds (e.g. glycinebetaine) and polyols (e.g. mannitol), as well as core metabolites such as proline and sucrose (Hu *et al.*, 2000).

Compatible solutes have been extensively researched and many transgenic plants have been made that over-accumulate these solutes (by stimulating synthesis, inhibiting breakdown or increasing transport to growing tissues). This work has been reviewed elsewhere (e.g. McNeil *et al.*, 1999; Chen and Murata, 2002; Garg *et al.*, 2002) and will not be discussed in detail here. With the notable exception of proline, transgenic manipulations do not usually lead to osmotically significant over-accumulation of metabolites (Chen and Murata, 2002), suggesting that the main mode of action of most manipulations made to date is not to balance

osmotic potential in the cytosol, thus enabling increased vacuolar salt accumulation. Instead, increased osmoprotectant accumulation may be protecting plants against damage, such as helping maintain protein structure or increasing scavenging of reactive oxygen species. Nevertheless, account of the metabolic costs of such manipulations must still be made (Huang *et al.*, 2000), and to minimize effects on the diversion of photosynthate to such solutes, their synthesis should be inducible.

Separating the effects of these compounds on salinity tolerance rather than drought tolerance has been difficult, especially when it is possible that the favourite model plant in which over-production has been generated (tobacco) may be more sensitive to the osmotic component of NaCl addition than to the ions themselves (Murthy and Tester, 1996). Nevertheless, in plants such as maize, there is a clear correlation between osmoprotectant production and salt tolerance (Saneoka *et al.*, 1995). It seems highly likely that these compounds are effective both in maintaining a negative osmotic potential in the cytoplasm and in directly protecting protein and ribosome structures from deleterious effects of elevated cytoplasmic Na<sup>+</sup>.

Although osmoprotectants alone could go some way to enabling a plant to tolerate salinity, significant amounts of Na<sup>+</sup> would still need to be compartmentalized. It is therefore likely that over-production of osmoprotectants would need to be accompanied by a pleiotropic up-regulation of Na<sup>+</sup> pumps (such as must occur for compatible solute synthesis in transgenic plants with elevated vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter activity; see above).

## 2. Tolerance of high cytoplasmic Na<sup>+</sup>

Cheeseman (1988) questioned whether Na<sup>+</sup> is necessarily toxic in the cytoplasm, a point supported by biochemical evidence showing that cytoplasmic reactions can appear to be tolerant of quite high concentrations of Na<sup>+</sup> (in the order of 100 mM) in the presence of compatible solutes, and even metabolic intermediates such as phosphoenolpyruvate (Shomer-Ilan *et al.*, 1991). There is evidence for the tolerance, *in vitro*, of cytoplasmic enzymes to high Na<sup>+</sup> in some salt-tolerant plants (e.g. of ribosomal activity; Flowers and Dalmond, 1992). However, because salt sensitivity of both ribosomes and enzymes can depend very much on relatively modest changes in the conditions used during the *in vitro* enzyme assays (e.g. Gibson *et al.*, 1984; Shomer-Ilan *et al.*, 1991; Solomon *et al.*, 1994), the relevance of any assay to the situation in the intact plant is uncertain. Attempts to identify biologically relevant tolerance of enzymes to high Na<sup>+</sup> would require complementation of *in vitro* assays with *in vivo* assays that also include concurrent monitoring of substrate levels and Na<sup>+</sup> concentrations.

However, in halophytic bacteria, where the structure of some enzymes is altered to provide unequivocal tolerance to high Na<sup>+</sup> (by, for example, having more acidic residues on the surface of the enzymes), enzymes become dependent on high concentrations of Na<sup>+</sup> for activity (Dym *et al.*, 1995). Thus, engineering of salt-tolerant enzymes in plants would not only require alteration of multiple proteins, but could

reduce metabolic efficiency in non-saline conditions. Thus, a more desirable strategy would be to alter concentrations of protective substances that help maintain protein structure in the face of elevated concentrations of Na<sup>+</sup> (see 'Synthesis of compatible solutes', and below).

## 3. Damage response and repair

Increased synthesis of a wide variety of proteins occurs in response to salt stress. Many of these, such as the osmotins and dehydrins, have properties similar to chaperones, and appear to be involved in the maintenance of protein structure in the face of elevated salts and other conditions in which protein–water interactions are disrupted (Ingram and Bartels, 1996; Campbell and Close, 1997). They may also act to stabilize membrane structure. These proteins are often highly hydrophilic and structured as random coils. Many of these stress-response proteins are probably elicited by the sudden osmotic shock of the experimental treatment. Others are probably involved mainly in plant responses to the osmotic component of high salinity. Detection of stress response proteins specific to Na<sup>+</sup> damage requires suitable osmotic controls and could, perhaps, be distinguished, at least in the shoot, by a relatively late appearance in the time course of Na<sup>+</sup> treatment (days to weeks after NaCl introduction) (Munns, 2002). Constitutive overexpression of a late embryogenesis abundant protein (LEA) from barley conferred tolerance to salt stress in transgenic rice (Xu *et al.*, 1996), although similar effects upon addition of mannitol suggest that the LEA was protecting against the osmotic effects of NaCl, rather than the ion-specific component. Constitutive overexpression in tobacco of a heat shock protein from the halotolerant cyanobacterium, *Aphanothece halophytica*, increased tolerance to high salinity and reduced shoot Na<sup>+</sup> concentrations, whilst having no measurable effect on unstressed plants (at least in the growth conditions employed in the experiments) (Sugino *et al.*, 1999). However, the lack of osmotic controls makes it difficult to assess whether the effects were ion-specific.

The osmoprotectant glycinebetaine has also been suggested to act as a chaperone, as well as potentially reducing lipid peroxidation and protecting mitochondrial electron transport reactions (Chen and Murata, 2002).

Synthesis of polyamines (such as putrescine and spermine) increases greatly in response to stresses, including salinity (Galston and Sawhney, 1990; Mansour, 2000), and the increase in putrescine levels (especially) appears to be greater in salt-tolerant than salt-sensitive lines of tomato (Santa-Cruz *et al.*, 1999). The function of these compounds is presumed to be protective, with a role in scavenging free radicals being a favoured hypothesis (Mansour, 2000). However, Lefevre *et al.* (2001) found an ion-specific increase in putrescine and tyramine concentrations which was higher in the roots of a salt-tolerant cultivar of rice (Pokkali) than in a salt-sensitive cultivar. However, the opposite trend was found in the shoots, perhaps reflecting different functions of these polyamines in roots and shoots.

Minimization of the generation of reactive oxygen species (ROS) as a result of inhibition of photosynthesis, and/or maximization of their removal ('scavenging') is

likely to be an important component of plant responses to high salinity, among other stresses (Zhu, 2001). Interestingly, activities of enzymes involved in reactive oxygen scavenging were higher in a salt-tolerant species of tomato than in its salt-sensitive cultivated relative (Shalata and Tal, 1998). This suggests that ROS scavenging activity was part of the active salt tolerance mechanisms of this species, rather than a secondary response to damage arising from salt stress (such as mediated by the osmotic component of salt stress).

Reactive oxygen species are also scavenged by osmoprotectants, such as proline and mannitol, and the role of these and other ROS scavenging systems is reviewed elsewhere (Xiong *et al.*, 2002). Although, intuitively, it might be more sensible to minimize damage rather than maximize its repair, it is notable that one of the few Na<sup>+</sup>-tolerant mutants identified to date appears to be tolerant owing to mutation of a negative regulator of enzymes involved in scavenging reactive oxygen species (Tsugane *et al.*, 1999). Although elevated levels of ROS may be a result primarily of osmotic stress causing reduced stomatal conductance (unfortunately not directly tested in the paper), that the mutation also provided some protection against low concentrations of LiCl suggests there was a substantial ion-specific component to the effect.

The identification of a salt-sensitive mutant through a screen for UV-sensitivity suggests that components of salinity tolerance may also be related to processes of DNA repair (Albinsky *et al.*, 1999).

#### 4. Genomic-scale observations of alterations in expression of genes

In the current scramble to employ microarrays to study changes in gene transcription, a wealth of data is emerging on effects of salinity on gene expression (Bohnert *et al.*, 2001; Kawasaki *et al.*, 2001; Seki *et al.*, 2001; Chen *et al.*, 2002; Ozturk *et al.*, 2002). Experiments need to be done in a physiologically realistic manner (e.g. to minimize the measurement of shock responses rather than adaptive responses to NaCl), and time courses will also be invaluable in enabling separation of primary damage limitation from secondary damage repair and acclimation. The relevance to salt-tolerance of studying responses in a salt-sensitive plant also needs to be considered. From the experiments published to date, it appears as if salinity affects the level of transcription of approx. 8 % of all genes. About 70 % of genes whose transcription is altered by salinity are distinct from those altered in response to drought stress, and distinct genes are activated depending on the tissue, developmental stage, and the rate and extent of onset of the stress (Bohnert, 2001). Consistent with this observation are data from differential display-PCR techniques suggesting that most salt-induced changes in gene expression are ABA-independent (Wei *et al.*, 2000). Similar differential subtraction techniques to compare expression in wild-type and *sos* mutants of arabidopsis in control and stressed conditions identified 84 salt-regulated genes, six of which are controlled by the SOS signalling pathway (which is described in more detail below) (Gong *et al.*, 2001).

Such data sets can be reconciled, at least in part, with older data reporting changes both in levels of protein expression in response to salinity and biochemical measurements of changes in enzyme activities. Interestingly, correlations made to date of responses in different varieties displaying different tolerances to salinity suggest that tolerance is most associated with the early responses to salinization, which include transcription of genes involved in signal transduction (Bohnert, 2001; Bohnert *et al.*, 2001; Kawasaki *et al.*, 2001). Such conclusions need to be reconciled with physiological experiments suggesting that physiological responses immediately after Na<sup>+</sup> shock are of little relevance to long-term responses to Na<sup>+</sup> (Munns, 1993, 2002). However, the treatments used in these microarray studies were extreme and could potentially lead to death of even tolerant plants over several days. For example, the description by Kawasaki *et al.* (2001) of irreversible wilting and death upon addition of 150 mM NaCl is not reassuring. As such, lack of differences after longer time intervals may reflect more the general effects of severe stress—more experiments need to be done using much lower concentrations of NaCl. The apparent lack of osmotic controls in many experiments also makes it difficult to separate Na<sup>+</sup>-specific effects from osmotic effects. Major bioinformatic analyses are now required to identify signalling pathways from such data and to predict likely biochemical consequences of these changes in transcription. Confirmation of such predictions with proteomic and biochemical studies directed by information gained from such transcriptomic studies will be necessary. Despite the likelihood that alternative splicing of mRNA may not be used as widely in plants as it is in animals (Arabidopsis Genome Initiative, 2000), the recent observation of the possible importance of pre-mRNA splicing in Na<sup>+</sup> tolerance also needs to be considered (Forment *et al.*, 2002).

#### 5. Signalling pathways

The effects of salt stress include both salt-specific and osmotic components, the latter including effects mediated by abscisic acid. These pathways, from the receptor perceiving salt stress to alteration of protein activity and gene transcription via signalling intermediates and phosphoprotein cascades have been reviewed extensively elsewhere (e.g. Leung and Giraudat, 1998; Hasegawa *et al.*, 2000; Schroeder *et al.*, 2001; Xiong *et al.*, 2002; Zhu, 2002). We shall add only a few comments to these more detailed accounts.

The relevance to field situations of many studies of salinity signalling may well be compromised because responses are often measured over a short time period after a rapid and large change in salinity. Thus, how much the responses relate to a shock response rather than physiologically relevant responses to elevated salinity is uncertain (see also Munns, 2002).

*Cytosolic calcium activity.* Although the mechanisms of signal perception remain unknown, and may involve proteins such as stretch-activated channels and ion-specific

receptors, it is clear that an early response to the sudden addition of extracellular Na<sup>+</sup> is a transient rise in cytosolic Ca<sup>2+</sup> (Knight *et al.*, 1997). However, the relevance of such rapid rises to plant responses in a fairly constant saline environment is uncertain, although salinity-induced changes in activity of Ca<sup>2+</sup> transporters and components of Ca<sup>2+</sup>-related signal transduction pathways suggest that Ca<sup>2+</sup> plays a physiologically relevant role in plant responses to salinity. For example, InsP<sub>3</sub>-activated Ca<sup>2+</sup> channel activity is induced by hypotonic pre-treatment (Allen and Sanders, 1994) and transcription of a gene encoding a phosphatidylinositol-specific phospholipase C also occurs upon salinization, consistent with a role for inositol phospholipid-based signalling responses in salinity responses (Hirayama *et al.*, 1995). Up-regulation by salt stress of expression of an endoplasmic reticulum (ER) Ca<sup>2+</sup>-translocating ATPase (Wimmers *et al.*, 1992) may also indicate an ongoing role for Ca<sup>2+</sup> in plant responses to salinity, as does the effects of SOS3, a Ca<sup>2+</sup>-binding protein, on salinity tolerance (see 'sos mutants').

*Protein phosphorylation and dephosphorylation.* There is extensive evidence for the central importance of protein phosphorylation and dephosphorylation in plant responses to salinity stress (Xiong *et al.*, 2002). It is also possible that membrane-bound histidine kinases could act as Na<sup>+</sup> sensors, as might occur in *Synechocystis* (Mikami *et al.*, 2002). In arabidopsis, overexpression of a GSK1/shaggy-like protein kinase caused plants to show symptoms similar to those of salt stress in unstressed conditions (anthocyanin synthesis, transcription of some NaCl stress-responsive genes), but also increased tolerance to salt stress (Piao *et al.*, 2001). These plants also accumulated higher concentrations of Na<sup>+</sup> in the shoot, as is observed in plants overexpressing a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter (see 'Pumping Na<sup>+</sup> into the vacuole'). It is possible that the increased kinase activity directly or indirectly increases activity of this Na<sup>+</sup> pump. Overexpression of a Dbf2 kinase also conferred tolerance to salinity (among other stresses) in arabidopsis suspension cells (Lee *et al.*, 1999).

There are myriad kinases and phosphatases in plants likely to be related to salinity responses and/or tolerance. These include metabolic phosphatases (e.g. HAL2; Gil-Mascarell *et al.*, 1999), Ca<sup>2+</sup>-dependent protein kinases (CDPKs), serine/threonine protein kinases (e.g. SOS2; Halfter *et al.*, 2000), mitogen-activated protein kinase (MAPK) cascades (Kovtun *et al.*, 2000; Tena *et al.*, 2001), two component histidine kinases (Urao *et al.*, 1999), Ca<sup>2+</sup>/calmodulin-activated serine/threonine-specific protein phosphatases (Pardo *et al.*, 1998; Kudla *et al.*, 1999), and so on. Determining how these interact to direct appropriate responses to salt stress will be a major undertaking requiring an integration of genomic and biochemical techniques with a framework of network analyses, such as speculated upon by Trewavas and Malho (1997) and Knight and Knight (2001).

*The sos mutants.* The *sos* (salt overly sensitive) mutants are a set of recessive mutants with increased sensitivity to

NaCl. They were identified by an inability to maintain root growth in elevated NaCl, and the defect appears to be NaCl-specific rather than osmotic because the mutants are specifically sensitive to NaCl and LiCl but not to mannitol. *sos* mutants have provided significant insights into components of signal transduction pathways that are likely to be involved in controlling plant responses to salinity. At least one of the proteins (SOS1) involved in the SOS signalling pathway shows putative cell-specific expression, indicating that these signalling pathways are involved in the regulation of whole plant Na<sup>+</sup> transport. We will discuss the proposed SOS signal transduction mechanisms here, but will refer to cell-specific processes affecting plant distribution of Na<sup>+</sup> in later sections.

SOS3 is a myristoylated calcium-binding protein that is thought to respond to salt-induced cytosolic Ca<sup>2+</sup> elevations (Liu and Zhu, 1997; Ishitani *et al.*, 2000). SOS3 interacts directly with SOS2, a serine/threonine protein kinase (Halfter *et al.*, 2000). One of the targets of this signalling pathway is SOS1, an Na<sup>+</sup>/H<sup>+</sup> antiporter localized to the plasma membrane (Shi *et al.*, 2002). SOS2 appears both to increase *SOS1* transcription and to increase the activity of the SOS1 protein through a direct protein-protein interaction (Shi *et al.*, 2000; Qiu *et al.*, 2002; Quintero *et al.*, 2002). In addition to its role as an Na<sup>+</sup> transporter, SOS1 has a large, putatively cytosolic domain which has been suggested to comprise an Na<sup>+</sup> sensor, raising the possibility of feedback by SOS1 on the SOS signalling pathway (Zhu, 2002). Promoter-GUS fusions indicated localization of *SOS1* transcripts in the epidermal and stelar cells of roots, suggesting a role in either removal of Na<sup>+</sup> from or loading of Na<sup>+</sup> into the xylem (Shi *et al.*, 2002) or phloem.

*sos1*, *sos2* and *sos3* mutants demonstrate different phenotypes with respect to Na<sup>+</sup> accumulation, indicating that SOS2 and SOS3 regulate the expression and/or activity of other Na<sup>+</sup>-related proteins in addition to SOS1 (Zhu *et al.*, 1998). *sos2* and *sos3* mutants accumulate more Na<sup>+</sup> than the wild type, whereas *sos1* mutants accumulate less, whilst still showing strong symptoms of Na<sup>+</sup> toxicity. The *sos1*, *sos2* and *sos3* mutants were initially identified in a screen for K<sup>+</sup> transport mutants, and all show defects in K<sup>+</sup> nutrition, being unable to grow normally in media with less than 20 mM K<sup>+</sup>. The interaction between Na<sup>+</sup> toxicity and K<sup>+</sup> deficiency in *sos* mutants has not yet been established, although the lack of K<sup>+</sup> transport activity by SOS1 when expressed in yeast suggests that the effects of the *sos1* mutation on K<sup>+</sup> transport are indirect (Quintero *et al.*, 2002). *sos4* encodes a pyridoxal kinase that is important for the synthesis of pyridoxal-5-phosphate. It is speculated that this may be involved in regulation of SOS1 activity because SOS1 contains a putative pyridoxal-5-phosphate binding motif in the C-terminal cytoplasmic tail (Zhu, 2002).

It is curious that despite evidence for the existence of other components of the *sos* signalling pathway, only four *sos* mutants have been identified, despite the size of the screen (confirmed by the high frequency of identification of alleles of most of the four *sos* mutants) (Zhu *et al.*, 1998). Moreover, a screen for knockout mutations complementing *sos3* Na<sup>+</sup> sensitivity identified multiple mutations of HKT1 and no other genes (Rus, 2001b). This may reflect the use of

root growth assays over relatively short periods (7 d) to identify the *sos* mutants, which may not detect differences in Na<sup>+</sup> transport that take longer to manifest and primarily affect shoot growth. For instance, *sos2* mutants showed almost no root growth in 50 mM NaCl, yet showed 100 % survival in 100 mM NaCl, whereas *sos3* mutants showed less than 50 % survival in 100 mM NaCl yet were considered less sensitive to NaCl than *sos2* mutants on the basis of root growth inhibition (Zhu *et al.*, 1998). *sos1*, *sos2* and *sos3* mutations all affect K<sup>+</sup> nutrition, and sensitivity to inhibition of root growth by NaCl was correlated with tissue [K<sup>+</sup>] but not [Na<sup>+</sup>] (Zhu *et al.*, 1998). A screen for survival, shoot size or leaf [Na<sup>+</sup>] at high salinity might pick out other mutants of relevance to Na<sup>+</sup> toxicity and Na<sup>+</sup> transport.

Related to the SOS pathway, the observation by Pandey *et al.* (2002) that increased expression of a protozoan calcium-binding protein in tobacco increased tolerance to salinity is also of interest, although how much of this tolerance is due 'simply' to increased vigour of the transgenic in control conditions is uncertain.

*Transcription factors.* The response to high salinity requires the coordinated induction of transcription of many genes, thus requiring the activity of specific sets of transcription factors and their binding to specific sequences in the promoter regions of target genes (Chen *et al.*, 2002; Xiong *et al.*, 2002). Such promoter regions include dehydration-responsive elements ('DREs') and ABA-responsive elements ('ABREs'), which are likely to be involved in plant responses to the osmotic effect rather than an Na<sup>+</sup>-specific effect. Various proteins binding to such elements have been identified using yeast one-hybrid screening (e.g. Liu *et al.*, 1998; Uno *et al.*, 2000). Constitutive overexpression of some of the genes encoding these proteins can induce strong constitutive overexpression of several stress-inducible genes, along with increased tolerance to abiotic stresses (Liu *et al.*, 1998). Although this increased tolerance was associated with reduced growth in unstressed conditions, placing such genes behind a strong stress-inducible promoter reduced this growth inhibition in unstressed conditions (Kasuga *et al.*, 1999).

Thus, by analysing information coming from microarray studies (e.g. Chen *et al.*, 2002), there are many possibilities for future identification of promoter elements that are specific to controlling gene expression in response to salinity stress. Identification of transcription factors binding to these elements will then enable their inducible overexpression in transgenic plants, potentially increasing salinity tolerance.

*Cell-specific signalling responses.* It is also important in these studies to try to differentiate responses that are relevant to a positive response of the plant towards tolerance of salinity, as opposed to responses that are related to a negative spiral towards death. To put this another way, genes related to damage limitation are more important than those related to a plant's attempts at damage repair. Such differences are best separated by the judicious use of time courses and different concentrations of NaCl. Another

approach could be to identify responses in particular cell types, with cell type-specific responses being more likely to be involved in damage limitation, whereas constitutive responses are more likely to be related to damage repair.

In this review, signalling has been discussed in the context of cellular adaptations to salinity because, at present, very little is known about cell-specific signalling responses to salinity. Most workers look at gene expression, Ca<sup>2+</sup> changes, etc. in whole organs, or even whole plants. The study by Kiegle *et al.* (2000) is the only one of which we are aware in which an attempt has been made to compare responses in different cell types, although this was limited to measurements of changes in cytosolic Ca<sup>2+</sup> in response to the sudden addition of large amounts of Na<sup>+</sup>. Thus, although distinctive oscillations in cytosolic Ca<sup>2+</sup> were observed in endodermal and pericycle cells, which have distinct functions in plant responses to salinity (as explained below, see 'Control of xylem loading' and 'Retrieval from the xylem'), more refined experiments must be performed to test the relevance of such observations to physiologically realistic conditions.

#### *Whole plant adaptations to high salinity*

In addition to features of every cell within the plant that promote cellular survival and thus contribute to the tolerance of the whole plant to salinity stress, plants can also have a wide range of other mechanisms that involve particular activities of specific cell types. These generally involve the control of uptake of Na<sup>+</sup> and of its distribution within the plant (Fig. 2). This includes processes such as (1) regulation of Na<sup>+</sup> delivery to the shoot, a process comprising several steps, namely: initial entry into root epidermal and cortical cells, a balance between influx and efflux; loading to the xylem; retrieval from the xylem before reaching the shoot (and storage in, e.g. pith cells). (2) Recirculation out of the shoot in the phloem. (3) Allocation to particular parts of the shoot (e.g. old leaves). (4) Secretion onto the surface of the leaf. (5) Control of transpiration. Any or all of these mechanisms may operate in a particular plant. We shall now consider each in turn.

##### *1. Regulation of Na<sup>+</sup> transport to the shoot*

Many of the mechanisms that enable plants to tolerate high soil salinity involve maintenance of low Na<sup>+</sup> in shoots, as evidenced by the frequently observed inverse correlation between amounts of shoot Na<sup>+</sup> and growth. Within crop species, it is often the case that varieties that are more tolerant of salinity have smaller amounts of salt reaching the shoot, or at least accumulating in the shoot. However, this relationship does not necessarily hold among species; for instance, barley and cotton are relatively salt-tolerant crop species, and tolerate higher shoot Na<sup>+</sup> concentrations than, for instance, wheat.

Concomitant with the maintenance of low shoot Na<sup>+</sup> is the maintenance of high K<sup>+</sup>. In fact, it is possible that a high K<sup>+</sup> : Na<sup>+</sup> ratio is more important for many species than simply maintaining a low concentration of Na<sup>+</sup> (Gorham *et al.*, 1990; Dubcovsky *et al.*, 1996; Maathuis and

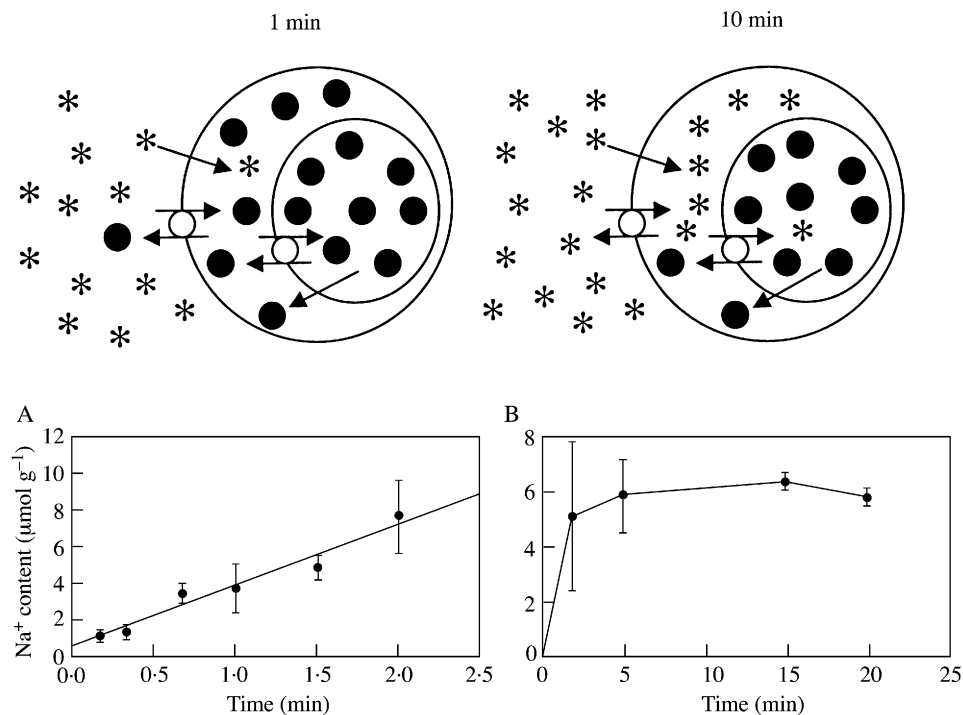


FIG. 3. Radioactive tracers can be used to measure unidirectional fluxes into cells at steady state. Plant root cells pre-treated with NaCl display bidirectional movement of  $\text{Na}^+$ , and measures of accumulation of chemical  $\text{Na}^+$  over time indicate only the net uptake rate (influx minus efflux). By labelling the external NaCl solution with  $^{22}\text{Na}^+$ , it is possible to separate influx and efflux. However, unidirectional fluxes can be measured only when there is a large difference in radioactive labelling between the compartments. For example, after 1 min of exposure to a  $^{22}\text{Na}^+$ -spiked NaCl solution some  $^{22}\text{Na}^+$  (asterisks) has entered the cell but the cytosol still contains mainly unlabelled  $\text{Na}^+$  (closed circles) and so there is negligible efflux of  $^{22}\text{Na}^+$  to the external solution (and to the vacuole). In this phase, accumulation of  $^{22}\text{Na}^+$  is linear with time (A), and provides a measure of unidirectional influx. The cytosol fills rapidly with  $^{22}\text{Na}^+$  because exchange with the external solution is rapid (influx of labelled  $\text{Na}^+$  and efflux of unlabelled  $\text{Na}^+$ ) and the cytosolic volume is small. As the proportion of  $^{22}\text{Na}^+$  to unlabelled  $\text{Na}^+$  rises in the cytosol, efflux of  $^{22}\text{Na}^+$  increases and the rate of accumulation of  $^{22}\text{Na}^+$  ceases to be linear and is no longer a measure of unidirectional influx (10 min, B). Exchange of  $\text{Na}^+$  between the cytosol and the vacuole is slower than with the external solution and so the vacuole continues to fill with  $^{22}\text{Na}^+$  after the cytosol has reached equilibrium with the external solution. Note that the radioactively labelled solution of NaCl is depicted as comprising  $^{22}\text{Na}^+$  only; however, the solution would contain only a very small proportion of radioactive  $\text{Na}^+$ . Data represent  $\text{Na}^+$  transport into roots of arabidopsis seedlings; from Essah (2000).

Amtmann, 1999; Cuin *et al.*, 2003), which makes sense given that much of the basis for  $\text{Na}^+$  toxicity is due to competition with  $\text{K}^+$  for  $\text{K}^+$ -binding sites (see 'The main bases for  $\text{Na}^+$  toxicity'). In this context, it is interesting that the *sos1*, *sos2* and *sos3* mutants are defective in  $\text{K}^+$  nutrition as well as  $\text{Na}^+$  transport. However, we will not discuss  $\text{K}^+$  specifically, except when it interferes directly with  $\text{Na}^+$ .

*Pathways for initial entry into the root.* It is clear that initial entry of  $\text{Na}^+$  from the soil solution into the root cortical cytoplasm is passive (Cheeseman, 1982), with  $\text{Na}^+$  influx being favoured energetically by differences in both concentration and voltage. Net accumulation in the plant is due to the balance between passive influx and active efflux. Exclusion of  $\text{Na}^+$  from the root at the site of initial entry has been suggested to be an important mechanism to minimize damage in saline soils for some plants (e.g. Schubert and Läuchli, 1990).

An important feature of the initial unidirectional entry of  $\text{Na}^+$  into roots is the very high rate of influx that has been measured. Unidirectional influx has been recorded as being in the order of 0.5–2.0  $\mu\text{mol g}^{-1}$  f. wt  $\text{min}^{-1}$  with 50 mM external  $\text{Na}^+$ , a value found in species as diverse as wheat

(Davenport, 1998), rice (L. Wang, R. Davenport and M. Tester, unpubl. res.) and arabidopsis (P. A. Essah, R. Davenport and M. Tester, unpubl. res.). This value is somewhat higher than those published previously (e.g. Zidan *et al.*, 1991; Elphick *et al.*, 2001), probably because the previous measures were influenced by significant efflux (due either to long periods of uptake or long rinse periods). We have undertaken high resolution time courses in each species, and these show clear non-linearities in influx in 5–10 min. Thus, very short exposures to  $^{22}\text{Na}^+$  are necessary to obtain an accurate measure of influx (Fig. 3). To put this high rate of influx into perspective, it can be calculated that without compensatory fluxes to the apoplast or vacuole, the cytoplasm of a cell containing no  $\text{Na}^+$  would equal an extracellular concentration of 50 mM within 3 min of being exposed to that concentration (assuming a cell diameter of 40  $\mu\text{m}$  and 10 % of the cell volume being cytoplasm). This corresponds to the range of time over which non-linearity in influx occurs, consistent with the need for very short influx times.

By using the very rough conversion of Pitman (1963) (that 1  $\mu\text{mol g}^{-1} \text{h}^{-1} \approx 3 \text{ nmol m}^{-2} \text{s}^{-1}$ ), an influx of 1  $\mu\text{mol g}^{-1}$  root f. wt  $\text{min}^{-1}$  equates to a flux per unit area of root plasma

membrane of 180 nmol m<sup>-2</sup> s<sup>-1</sup>, or a current density of 34 mA m<sup>-2</sup>. This is of a similar magnitude to Na<sup>+</sup> inward currents at physiologically reasonable potentials measured in patch clamped protoplasts isolated from roots of maize (Roberts and Tester, 1997), wheat (Tyerman *et al.*, 1997; Buschmann *et al.*, 2000), rye (White and Lemtiri-Chlieh, 1995) and arabidopsis (Demidchik and Tester, 2002).

These high rates of unidirectional influx of Na<sup>+</sup> do not result in rapid accumulation of Na<sup>+</sup>. Root Na<sup>+</sup> concentrations tend to remain fairly constant over time in saline conditions, and shoot Na<sup>+</sup> content tends to rise rather slowly. Thus, the net rate of uptake of Na<sup>+</sup> is usually much lower than the unidirectional influx measured by short-term uptake of radioactive tracers or patch-clamp measurements, implying substantial efflux of Na<sup>+</sup> across the plasma membrane. This efflux must be energetically expensive (see 'Na<sup>+</sup> efflux pathways', below).

Interestingly, the few measurements of which we are aware suggest that unidirectional influx into halophyte roots is much lower than that into roots of glycophytes. Cheeseman *et al.* (1985) measured <sup>22</sup>Na<sup>+</sup> uptake into roots of the halophytic dicotyledon, *Spergularia marina*. Uptake was linear from 45 s to 240 min, with no evidence of efflux. This suggested, remarkably, that the plant might have been able to control Na<sup>+</sup> influx, and hence net accumulation, without requiring significant Na<sup>+</sup> efflux. The rate of unidirectional Na<sup>+</sup> uptake from 100 mM NaCl was 0.24 μmol g<sup>-1</sup> root f. wt min<sup>-1</sup>, compared with 1.5 μmol g<sup>-1</sup> root f. wt min<sup>-1</sup> in wheat roots. Indirect measures of unidirectional influx of Na<sup>+</sup> have been obtained from efflux analysis in several halophytic monocotyledons. Jefferies (1973) gave two estimates of influx into *Triglochin maritima* roots from 100 mM NaCl of 0.065 and 0.21 μmol g<sup>-1</sup> root f. wt min<sup>-1</sup>, with similarly low rates of efflux. Na<sup>+</sup> influx into *Eleocharis uniglumis* roots in 74 mM NaCl was estimated to be 0.13 μmol g<sup>-1</sup> root f. wt min<sup>-1</sup> (although the higher rate of efflux suggest that these data are unreliable) (Shepherd and Bowling, 1979). Other measures of Na<sup>+</sup> accumulation in halophytes have involved very low concentrations of NaCl in the influx medium (e.g. 10 mM). At such low concentrations, halophytes may demonstrate higher rates of uptake than glycophytes, possibly because high Na<sup>+</sup> uptake at these non-toxic levels of Na<sup>+</sup> represents a constitutive drought-tolerance response. It would be interesting to see more measurements of unidirectional and net uptake of Na<sup>+</sup> into halophytes at concentrations of NaCl that are toxic to glycophytes. Halophytes are better at regulating Na<sup>+</sup> transport than glycophytes, and this control may be exerted in part by more effective prevention of initial Na<sup>+</sup> entry.

We consider in this review three pathways for Na<sup>+</sup> influx. Two protein-mediated pathways can be distinguished by their sensitivity to addition of extracellular Ca<sup>2+</sup>, and a third pathway appears to be due to 'leakage' into the root via the apoplast. The relative contribution of each of these pathways varies with species and growth conditions. Although it is most likely that each of these pathways is structurally independent, it is conceivable that one structure could account for more than one type of pathway. For example, Davenport and Tester (2000) proposed that the

partial sensitivity to Ca<sup>2+</sup> of a non-selective cation channel in wheat roots provided evidence consistent with these channels being the primary pathway for both Ca<sup>2+</sup>-sensitive and Ca<sup>2+</sup>-insensitive components of protein-mediated Na<sup>+</sup> influx.

Identification of the genes disrupted in Na<sup>+</sup>-accumulating mutants, such as described by Nublat *et al.* (2001), will provide further insights into the molecular basis for these pathways in the near future. However, identification of Na<sup>+</sup>-excluding mutants would be of more interest for both intellectual reasons (as described above) as well as for the obvious applied benefits arising from Na<sup>+</sup> exclusion.

*i. The Ca<sup>2+</sup> sensitive pathway.* It has long been known that toxic effects of Na<sup>+</sup> can (usually) be ameliorated by addition of up to 10 mM Ca<sup>2+</sup> to the external solution. Although the effects of Ca<sup>2+</sup> are likely to be complex (Cramer, 2002), its protection against Na<sup>+</sup> is due, at least in part, to an inhibition of the accumulation of Na<sup>+</sup> into the roots and shoots of plants (and a stimulation of the accumulation of K<sup>+</sup>). This reduced accumulation of Na<sup>+</sup> is at least partly due to a partial inhibition of the unidirectional influx of Na<sup>+</sup> into the roots by Ca<sup>2+</sup>. Interestingly, it is also observed that Ca<sup>2+</sup> reduces Na<sup>+</sup>-stimulated K<sup>+</sup> (<sup>86</sup>Rb<sup>+</sup>) efflux (Cramer *et al.*, 1985; S. Shabala, pers. comm.). As an aside, an important consideration when studying the interactions of Ca<sup>2+</sup> and Na<sup>+</sup> is the effect of NaCl on Ca<sup>2+</sup> activity (Cramer and Lauchli, 1986) (Fig. 4). Apparent competitive effects of NaCl on Ca<sup>2+</sup> binding to proteins, cell membranes or cell walls, or on Ca<sup>2+</sup> transport or nutrition must be distinguished from the simple reduction of Ca<sup>2+</sup> activity in solutions with high ionic strength.

The effect of extracellular Ca<sup>2+</sup> on Na<sup>+</sup> and K<sup>+</sup> transport has recently been attributed to the activity of the SOS signalling pathway (Liu and Zhu, 1998). According to this

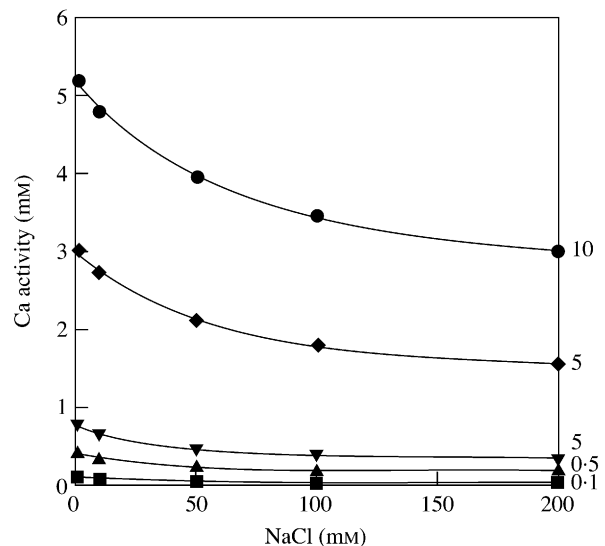


FIG. 4. The decrease in Ca<sup>2+</sup> activity with increasing concentrations of NaCl (calculated using GEOCHEM-PC version 2.0; Parker *et al.*, 1995).

model, salinity causes a rise in cytosolic Ca<sup>2+</sup>, which activates SOS3, leading to changes in expression and activity of Na<sup>+</sup> and K<sup>+</sup> transporters. The extent of the rise in cytosolic Ca<sup>2+</sup> is thought to depend on the concentration of extracellular Ca<sup>2+</sup>. *sos3* mutants express a mutant form of the SOS3 protein that is postulated to be less sensitive to Ca<sup>2+</sup>, and *sos3* mutants require higher concentrations of extracellular Ca<sup>2+</sup> than wild type arabidopsis for root elongation in NaCl. This hypothesis could be tested by using SOS3 knockout plants to check whether absence of SOS3 makes the plants insensitive to extracellular Ca<sup>2+</sup>, as would be predicted by the SOS model. Another possibility is that extracellular Ca<sup>2+</sup> alters Na<sup>+</sup> influx rates directly, and it is cytosolic Na<sup>+</sup> levels that activate the SOS pathway via SOS3 (possibly by a rise in cytosolic Ca<sup>2+</sup>). In wheat, extracellular Ca<sup>2+</sup> inhibits unidirectional Na<sup>+</sup> influx and also inhibits Na<sup>+</sup> influx through a non-selective cation channel isolated in planar lipid bilayers, suggesting that the effect of Ca<sup>2+</sup> on Na<sup>+</sup> influx may be a direct one not requiring cytosolic signalling for modification of ion channel activity (Davenport and Tester, 2000). Similar effects of extracellular Mg<sup>2+</sup> on Na<sup>+</sup> influx strengthen this hypothesis (Davenport and Tester, 2000).

It has been argued elsewhere that the most likely pathway for this important Ca<sup>2+</sup>-sensitive Na<sup>+</sup> influx is non-selective cation channels (Amtmann and Sanders, 1999; Tyerman and Skerrett, 1999; White, 1999; Davenport and Tester, 2000; Demidchik *et al.*, 2002). However, although there are many candidate genes that could encode these non-selective cation channels, their precise molecular identity remains obscure (Demidchik *et al.*, 2002). Two major candidates for non-selective cation channels are the cyclic nucleotide-gated channels (the CNGCs) and the putative glutamate-activated channels (the GLRs). That these genes can encode channels with properties of non-selective cation channels has been demonstrated (CNGCs, Leng *et al.*, 2002; GLRs, Cheffings, 2001; Lacombe *et al.*, 2001), but the function *in planta* of particular gene products remains uncertain until the membranes to which the gene products are targeted and the cell types in which they are expressed are established, and effects on influx in plants over- and underexpressing these genes are measured. Discrepancies also need to be resolved between the properties of currents arising from heterologous overexpression of these channels, and currents affected by cyclic nucleotides measured *in planta* (see below). Following the precedent from animal cells for both CNGCs and GLRs, co-expression for formation of heteromultimers may be important for determining selectivity and control of activity.

General correlative evidence for the importance of CNGCs in Na<sup>+</sup> influx is provided by the inhibition of Na<sup>+</sup> influx and of non-selective cation channel activity by extracellular addition of membrane-permeant analogues of cyclic nucleotides (Maathuis and Sanders, 2001; P. A. Essah, R. Davenport and M. Tester, unpubl. res.). However, the inhibitory effects of cyclic nucleotides *in planta* are in contrast with those on plant CNGCs expressed in heterologous systems, where cyclic nucleotides activate non-selective cation currents (Leng *et al.*, 2002). Likewise, involvement of GLRs in Na<sup>+</sup> influx is suggested by increases

in Na<sup>+</sup> influx and non-selective cation channel activity upon addition of glutamate (V. Demidchik, P. A. Essah and M. Tester, unpubl. res.). However, the GLRs characterized to date in heterologous systems show either no activity (AtGLR2-8) or glutamate-insensitive activity (AtGLR3-7 and AtGLR3-4).

*LCT1* from wheat encodes an unusual protein which, when expressed in yeast, causes an increased influx of a wide range of cations, as well as hypersensitivity to Na<sup>+</sup> (Schachtman *et al.*, 1997; Clemens *et al.*, 1998; Amtmann *et al.*, 2001). Importantly, Na<sup>+</sup> influx and salt sensitivity in yeast were reduced by addition of extracellular Ca<sup>2+</sup> (and K<sup>+</sup>), compatible with this protein product being involved in the Ca<sup>2+</sup>-sensitive Na<sup>+</sup> influx pathway. However, the selectivity of the cation uptake elicited by *LCT1* was very similar to that of native yeast cation transport (Amtmann *et al.*, 2001), raising the possibility that *LCT1* was acting to stimulate endogenous ion transport mechanisms in yeast. *LCT1* is thought to be localized to the yeast plasma membrane, but its activity and localization have not yet been characterized in plant systems.

It seems likely that within the complex and heterogeneous structure of a root, there exist several pathways for Ca<sup>2+</sup>-sensitive Na<sup>+</sup> influx, and that these could be encoded by members of more than one gene family.

*ii. Ca<sup>2+</sup>-insensitive pathways.* The Ca<sup>2+</sup>-insensitive component of Na<sup>+</sup> influx could be due, at least in part, to the Ca<sup>2+</sup>-insensitive component of influx through non selective cation channels (NSCCs). It is notable that inhibition by Ca<sup>2+</sup> of Na<sup>+</sup> currents through NSCCs is both partial and occurs with a very similar affinity to inhibition by Ca<sup>2+</sup> of Na<sup>+</sup> influx into roots (Davenport and Tester, 2000). However, there are also other possible pathways, most notably encoded by members of the *HKT*, *KUP* and *HAK* gene families.

The *HKT1* transporter from wheat, when expressed in *Xenopus* oocytes and yeast, catalyses a high affinity Na<sup>+</sup>/K<sup>+</sup> symport and, in elevated Na<sup>+</sup>, can catalyse low affinity Na<sup>+</sup> uniport (Rubio *et al.*, 1995). This influx is apparently Ca<sup>2+</sup>-insensitive (Tyerman and Skerrett, 1999). The arabidopsis homologue appears to catalyse only Na<sup>+</sup> influx in *Xenopus* oocytes, but partially complemented a K<sup>+</sup>-deficient yeast mutant (Uozumi *et al.*, 2000). However, the membrane(s) to which *HKT1* is targeted in intact plants is uncertain.

A screen to identify gene knockouts that could complement the salt-sensitive mutation, *sos3*, found a series of independent transformants that all had reduced activity of *HKT1* (Rus *et al.*, 2001b). This suggests that this gene is involved in Na<sup>+</sup> influx, an observation that is compatible with the Na<sup>+</sup> transport activity of *HKT1* when overexpressed in heterologous systems (see above). However, whether the Na<sup>+</sup> transport capacity of *HKT1* is relevant in the entry of Na<sup>+</sup> across the plasma membrane of cells in intact roots remains uncertain. It is possible that these mutations are altering the ability of plants to coordinate different fluxes as the intracellular content rises. Zhu (2002) suggests that *HKT1* is also under the control of *SOS3*, and

would perhaps be suppressed under saline conditions in wild type plants.

*iii. Bypass flow.* A third pathway for  $\text{Na}^+$  entry into the plant appears to be due to 'leakage' into the root via the apoplast. There is little effect of external  $\text{Ca}^{2+}$  on salt tolerance of rice and on the uptake of  $\text{Na}^+$  (Yeo and Flowers, 1985; Yeo *et al.*, 1987), at least for the varieties and growth conditions used in studies to date. By using an apoplastic fluorescent dye, Yadav *et al.* (1996) and Yeo *et al.* (1999) have shown that rice plants with high shoot  $\text{Na}^+$  have high apoplastic water flow. The most likely explanation for these data is that most  $\text{Na}^+$  that enters the rice plant does so not through membranes, but instead enters through 'leaks' in the endodermis. This could occur at root branch points, root apices, or the uptake may be due to the intrinsic permeability of the physically intact, mature endodermis. The extent of the contribution of this apoplastic flow to total  $\text{Na}^+$  influx into the plant clearly varies with species; for example, Garcia *et al.* (1997) estimated that this 'bypass flow' in rice was about ten times greater than that in wheat.

The possible significance of the apoplastic pathway in whole plant  $\text{Na}^+$  uptake is supported by classical observations that halophytes have many anatomical adaptations to minimize apoplastic entry of salt into the plant. The width of the Casparian band is two to three times greater in halophytes than in non-halophytes (Poljakoff-Mayber, 1975), and the inner layer of cortical cells can differentiate into a second endodermis (Stelzer and Läuchli, 1977). More recently, in cotton seedlings, salinization has also been shown to accelerate formation of a Casparian strip and induce formation of an exodermis (Reinhardt and Rost, 1995).

*iv.  $\text{Na}^+$  efflux out of the root.* Net accumulation of  $\text{Na}^+$  into root cortical cells is due to a balance between influx through ion channels and efflux through a probable  $\text{Na}^+/\text{H}^+$  antiporter. A reduction in  $\text{Na}^+$  accumulation, and thus an increase in tolerance to salinity, can be due to either a decrease in influx or an increase in efflux. Efflux is a process that is probably catalysed by an  $\text{Na}^+/\text{H}^+$  antiporter (Blumwald *et al.*, 2000), although evidence for the existence of such activity has not always been found (Mennen *et al.*, 1990). However, whether such a lack of evidence indicates biological reality or experimental artefact can be difficult to determine conclusively. In arabidopsis, SOS1 has been demonstrated to be localized to the plasma membrane (Shi *et al.*, 2002), and plasma membrane vesicles from *sos1*, *sos2* and *sos3* mutants showed lower rates of  $\text{Na}^+$ -induced acidification compared with those of the wild type (Qiu *et al.*, 2002). This transporter has been proposed to operate in either influx or efflux of  $\text{Na}^+$ , depending on the  $\text{Na}^+$  concentration of the growth medium. This is discussed further below; see 'Retrieval from the xylem'.

An important question regarding  $\text{Na}^+/\text{H}^+$  antiporters is the stoichiometry of the exchange. An electroneutral exchanger would stall in saline conditions if external pH increased towards neutrality (Garcia-deblás *et al.*, 2001). More generally, stoichiometry will determine the metabolic cost of  $\text{Na}^+$

extrusion. A fixed stoichiometry of, for instance, 1 : 1 exchange of  $\text{Na}^+$  for  $\text{H}^+$ , would fix the cost at 1 ATP molecule hydrolysed per  $\text{Na}^+$  ion extruded (given a 1 : 1 ratio of ATP hydrolysis to  $\text{H}^+$  extrusion by the plasma membrane ATPase; Briskin *et al.*, 1991, 1995). However, the theoretical thermodynamic cost of  $\text{Na}^+$  extrusion (i.e. the energy required to move one  $\text{Na}^+$  ion from the cytosol to the external solution against the electrochemical potential difference) is likely to be much less than the energy expended in  $\text{Na}^+/\text{H}^+$  exchange, and will vary depending on external salinity, influx rate and intracellular transport. Thus, roots may be committed to an unnecessarily expensive mechanism of  $\text{Na}^+$  extrusion. This kind of problem has been suggested in explanation of ammonium toxicity in wheat, where the increase in respiration in high  $\text{NH}_4^+$  conditions far exceeds the theoretical cost of  $\text{NH}_4^+$  extrusion, and suggests a ratio of 1 ATP hydrolysed per  $\text{NH}_4^+$  ion extruded (Kronzucker *et al.*, 2001). Potentially significant is that the cost of vacuolar compartmentation of  $\text{Na}^+$  may be more flexible, given the variety and flexibility of tonoplast  $\text{H}^+$  pumps (Rea and Sanders, 1987).

Efflux from roots must be active, so the activity of primary (ATP-hydrolysing)  $\text{Na}^+$  pumps [such as found in many algae (Gimmler, 2000) and fungi (Benito *et al.*, 2002)] should also be considered. However, to date, analyses of the arabidopsis genome have not indicated the likelihood of  $\text{Na}^+$ -extruding ATPases in terrestrial plants (Garcia-deblás *et al.*, 2001). Nevertheless, expression of a fungal  $\text{Na}^+$ -extruding ATPase in root epidermal and cortical cells might increase salt tolerance, although the effects of the relatively poor  $\text{Na}^+/\text{K}^+$  selectivity of such pumps would need to be monitored.

Addition of  $\text{Na}^+$  increased mRNA levels encoding plasma membrane  $\text{H}^+$ -translocating ATPases in roots of tobacco and rice (Niu *et al.*, 1996; Zhang *et al.*, 1999). This may be related to an increased requirement for  $\text{H}^+$ -extrusion to sustain activity of the  $\text{Na}^+/\text{H}^+$  antiporter, although these have yet to be experimentally linked.

*v. Control of net uptake.* The rates of unidirectional influx measured in glycophytes can exceed net uptake by an order of magnitude or more, implying high rates of  $\text{Na}^+$  efflux (Jacoby and Hanson, 1985; Davenport *et al.*, 1997; P. A. Essah, R. Davenport and M. Tester, unpubl. res.). This raises two questions: why is the unidirectional influx rate so high, and what determines the net uptake rate?

High unidirectional influx is typical of glycophytes and occurs via mechanisms that are mostly not  $\text{Na}^+$ -selective (Demidchik *et al.*, 2002), suggesting that  $\text{Na}^+$  influx is largely an 'accidental' process. The transporters involved in  $\text{Na}^+$  influx probably usually function in uptake of other cations (or small solutes generally, in the case of apoplastic bypass flow), such as  $\text{NH}_4^+$  (White, 1996) or  $\text{Ca}^{2+}$  (White and Davenport, 2002). The normal physiological roles of these  $\text{Na}^+$  influx pathways remain largely unknown, and therefore it is not known whether the selectivity or activity of these mechanisms could be altered to reduce  $\text{Na}^+$  transport without detriment to other functions. It appears that in glycophytes, net  $\text{Na}^+$  uptake is regulated less by

control of influx than by efflux of excess  $\text{Na}^+$ , probably via  $\text{Na}^+$ -selective  $\text{Na}^+/\text{H}^+$  antiporters (Qiu *et al.*, 2002). An interesting question is why  $\text{Na}^+$  influx is not  $\text{Na}^+$ -selective yet efflux appears to be, and whether this is also the case in NaCl-grown halophytes.

The high rates of both influx and efflux of  $\text{Na}^+$  in plant roots then raises the question of how roots determine the rate of net influx. Net influx is not simply that by which efflux fails to keep pace of influx, because there is evidence for control of internal  $\text{Na}^+$  levels, at least in roots. For instance, root  $\text{Na}^+$  concentration is less variable than shoot  $\text{Na}^+$  concentration, and it often varies little among species or varieties differing greatly in shoot uptake of  $\text{Na}^+$  (rice and *Phragmites communis*, Matsushita and Matoh, 1991; barley: Wyn Jones and Storey, 1978; maize, Fortmeier and Schubert, 1995; wheat, Schachtman *et al.*, 1989; Gorham *et al.*, 1990; Santa-María and Epstein, 2001; Munns, 2002, unpublished data; chenopods, Reimann and Breckle, 1993). Moreover root  $[\text{Na}^+]$  is not linearly related to external  $[\text{NaCl}]$  and shows saturation at moderate external NaCl levels in some species and experimental conditions (unpublished data in Munns, 2002). This suggests the existence of a setpoint in roots that regulates root  $\text{Na}^+$  content and possibly also net uptake. This would imply that root cells sense internal  $\text{Na}^+$  levels and control  $\text{Na}^+$  transporters appropriately. Unlike shoots, roots can regulate their  $\text{Na}^+$  concentration by controlling the rates of efflux in two directions, both out of the root into the external solution, and into the xylem for transfer to the shoot. Thus, it is possible that export to the shoot could occur as a side-effect of root processes governing root  $\text{Na}^+$  homeostasis. For instance, in wheat, the inhibition of unidirectional  $\text{Na}^+$  influx by addition of external  $\text{Ca}^{2+}$  has a greater effect on the  $\text{Na}^+$  concentration of shoots than roots (Reid and Smith, 2000). This may be because the root  $\text{Na}^+$  concentration is controlled independently of the rate of influx, whereas  $\text{Na}^+$  transport to the shoot depends on the rate of efflux of excess root  $\text{Na}^+$ . Thus, although reducing  $\text{Na}^+$  influx into roots may have little effect on root  $\text{Na}^+$  concentrations, it could significantly reduce accumulation of  $\text{Na}^+$  in the shoot.

The high rates of  $\text{Na}^+$  influx and efflux also imply that small changes in either will cause large changes in net uptake. Consider two plants that have the same influx rate, but efflux  $\text{Na}^+$  at rates 90 % and 95 %, respectively, of the influx rate. The first plant will have double the net uptake rate of the second, although the plants differ trivially in  $\text{Na}^+$  transport rates. Furthermore, if these plants maintain the same root  $\text{Na}^+$  concentration, then shoot  $\text{Na}^+$  uptake will vary by a factor greater than two. Thus, very small differences in transporter activity may lead to large differences in shoot accumulation of  $\text{Na}^+$ .

*Control of xylem loading.* The prevention of  $\text{Na}^+$  accumulation in shoots by the maintenance of low  $\text{Na}^+$  concentrations in the xylem can be effected by minimization of  $\text{Na}^+$  entry to the xylem from the root symplast, or by maximization of retrieval back out from the xylem before it reaches sensitive tissues in the shoot. In the following discussion we assume that the xylem is an apoplastic

compartment; however, it is possible that xylem loading could take place into living xylem cells in the young parts of roots (McCully *et al.*, 1987). The rate of  $\text{Na}^+$  translocation was higher from younger parts of barley roots at low  $\text{Na}^+$  concentrations (0.2 mM) (Shone *et al.*, 1969); however, we know of no comparable data for higher concentrations of NaCl. If most  $\text{Na}^+$  uptake were in fact into living xylem then this would of course alter the considerations of membrane transport below, and create a spatial separation between the processes of xylem loading and  $\text{Na}^+$  retrieval from the xylem.

An example of the importance of the barrier at the xylem is illustrated by studies of the salt-exclusion abilities of a salt-tolerant wheat line and of an amphiploid cross between wheat and the salt-tolerant wheat grass, *Lophopyrum elongatum*. Exclusion of  $\text{Na}^+$  from the shoots of these lines could be attributed to prevention of  $\text{Na}^+$  entry into the xylem from the root cortex (Gorham *et al.*, 1990; Santa-María and Epstein, 2001). However, the mechanisms and controls of the processes of net  $\text{Na}^+$  efflux into the xylem are unknown.

An essential thesis of this review, and of the research in our laboratory, is that the management of  $\text{Na}^+$  movements within the plant requires specific cell types in specific locations within the plant catalysing transport in a coordinated manner (see also Tester and Leigh, 2001). For example, to minimize  $\text{Na}^+$  delivery to the shoot in the apoplastic compartment of the xylem, cells in the outer half of the root need to minimize influx from and/or maximize efflux to the soil solution; whereas in the inner half of the root, cells would need to maximize influx from and/or minimize influx to the xylem solution. Thus, to minimize delivery of  $\text{Na}^+$  to the shoot, plasma membrane transport processes in the outer and inner halves of the root (i.e. on either side of an apoplastic barrier, the endodermis or exodermis) would need to be manipulated in opposite directions. To further understand complex whole plant adaptations to salinity, more knowledge is required of cell-specific transport processes and the consequences of manipulation of transporters and signalling elements in specific cell types.

To begin to address the issue of what proteins could be responsible for the loading of  $\text{Na}^+$  into the xylem, it is useful to know the energetics of  $\text{Na}^+$  movement into the xylem. It is possible that the movement of  $\text{Na}^+$  into the xylem from stelar parenchyma cells is active. Although the difference in  $\text{Na}^+$  activity is uncertain, it may be quite small. In the xylem sap,  $\text{Na}^+$  concentrations appear to be in the order of 1–10 mM [as measured both in detopped plants oozing xylem sap (Rozema *et al.*, 1981; Munns, 1985; Shi *et al.*, 2002) and in xylem of transpiring plants accessed using spittlebugs (Watson *et al.*, 2001)]. Similar values (1–30 mM) appear to be found in the cytoplasm of root cells [as measured by x-ray microanalysis (Behl, cited in Jeschke 1984; Jeschke and Stelzer, 1976; Koyro and Stelzer, 1988) and ion-sensitive electrodes (Carden, 1999)]. If this is the case, the energy difference will be mainly influenced by the potential difference (PD) across the plasma membrane, which appears to be around 100 mV negative inside parenchyma

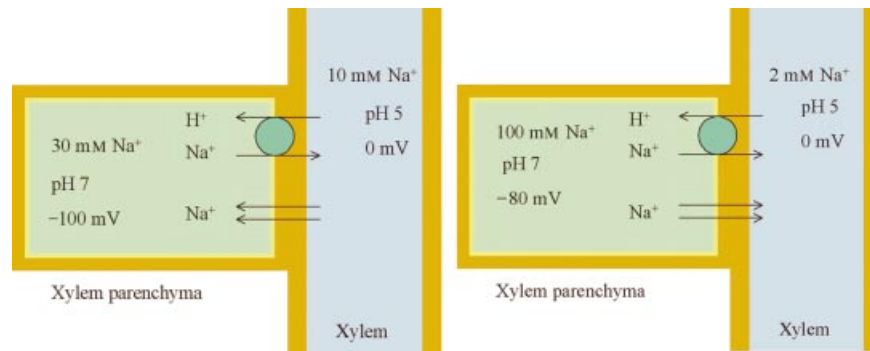


FIG. 5. Factors affecting the energetics of  $\text{Na}^+$  efflux into the xylem. Assuming a 1 : 1 stoichiometry for  $\text{Na}^+ : \text{H}^+$  exchange, then  $\text{Na}^+/\text{H}^+$  antiporters will transport  $\text{Na}^+$  into the xylem, due to the large pH difference between the cytosol and xylem. However, if the xylem pH changes, or if the stoichiometry of the antiporter is different, then antiporters could act to pump  $\text{Na}^+$  out of the xylem solution. If the intracellular concentration of  $\text{Na}^+$  is much higher than the xylem concentration, and if xylem parenchyma cells are slightly depolarized at high  $\text{NaCl}$ , then efflux to the xylem can occur passively via ion channels (right).

cells relative to the xylem sap (DeBoer, 1999; Wegner *et al.*, 1999) (Fig. 5).

In this situation,  $\text{Na}^+$  fluxes into the xylem will be active, which means that the (generally detrimental) accumulation of  $\text{Na}^+$  in shoots is being driven by a pump, not a passive leak. This posits the somewhat odd situation where detrimental accumulation of  $\text{Na}^+$  in the shoot is being driven by an active pumping of  $\text{Na}^+$  into the xylem in roots. Nevertheless, in arabidopsis, the *SOS1* gene encoding the putative  $\text{Na}^+/\text{H}^+$  antiporter was reported to be preferentially expressed at the xylem symplast boundary of roots (indicated by promoter-GUS fusions) (Shi *et al.*, 2002). At moderate  $\text{Na}^+$  concentrations, *SOS1* is proposed to function in active efflux of  $\text{Na}^+$  to the xylem (because *sos1* mutants accumulated less shoot  $\text{Na}^+$  than did the wild type). However, given that *SOS1* is also predicted to feed back on the *SOS2/SOS3* pathway, and possibly signal intracellular  $\text{Na}^+$  levels, it is likely that *sos1* mutants will differ from wild type in several aspects of  $\text{Na}^+$  transport, making elucidation of transporter function difficult. Nevertheless, it is credible that arabidopsis could actively accumulate  $\text{Na}^+$  in the shoot under moderate  $\text{Na}^+$  stress, perhaps in response to osmotic stress. Whether other species, in particular cereals, do this remains unknown.

It is also possible that  $\text{Na}^+$  transport into the xylem is passive, at least in rapidly transpiring plants. If cytosolic  $\text{Na}^+$  in stelar cells is higher than quoted above (say it is  $100 \text{ mM}$ , Hajibagheri *et al.*, 1985, 1987; Harvey, 1985) and if xylem  $\text{Na}^+$  is at the lower end of the above range (say,  $2 \text{ mM}$ , as measured in detopped barley under conditions mimicking high transpiration rates; Munns, 1985), then the energy difference could favour the passive leakage of  $\text{Na}^+$  into the xylem.

This discussion illustrates how little we know about the movement of  $\text{Na}^+$  into the xylem, with even the energetics of the process uncertain. This profound lack of knowledge is remarkable because these processes are clearly very important. It is, of course, possible that a false dichotomy is presented above, and  $\text{Na}^+$  loading could be active at low salinities and passive at high salinities, a possibility explored by Shi *et al.* (2002) and discussed further below.

The control of xylem loading by ABA is also relevant in these considerations. Although inhibition of channel-mediated efflux of  $\text{K}^+$  and  $\text{Cl}^-$  into the xylem by ABA has been well documented using both radioactive and electrophysiological techniques (Cram and Pitman, 1972; Roberts, 1998; Gilliam, 2002), there has been much less study of the control by ABA of the loading of  $\text{Na}^+$ . One interesting observation, however, is that addition of ABA stimulated  $\text{H}^+$  extrusion into the xylem (Clarkson and Hanson, 1986), a process that, naively, would tend to stimulate, rather than inhibit, any  $\text{Na}^+/\text{H}^+$  antiporter activity in the plasma membrane of xylem parenchyma cells.

There has also been more recent work regarding plasma membrane  $\text{H}^+$ -translocating ATPases that are localized to the endodermis. Addition of  $\text{NaCl}$  to the halophyte *Atriplex nummularia* increased mRNA levels of such an ATPase (Niu *et al.*, 1996), and a T-DNA knockout of an endodermal  $\text{H}^+$ -ATPase in arabidopsis increased the salt sensitivity and decreased the ability of plants to maintain low shoot  $\text{Na}^+$  in salinized conditions (Vitart *et al.*, 2001).

Another potential controller of  $\text{Na}^+$  loading is inositol, which has been found to stimulate  $\text{Na}^+$  transfer to the shoot in the salt-tolerant plant, *Mesembryanthemum crystallinum* (Nelson *et al.*, 1999). In the intact plant, inositol is synthesized in the leaf in response to salinization, and transported to the root in phloem. It is suggested that this acts as a long-distance signal of shoot water stress, with stimulation of  $\text{Na}^+$  uptake facilitating a lowering of the osmotic potential in the shoot.

Mutants of arabidopsis that accumulate high concentrations of  $\text{Na}^+$  have been identified by Nublat *et al.* (2001). In one of these, *sas1*, accumulation of  $\text{Na}^+$  in the shoot is increased two- to seven-fold, without affecting root  $\text{Na}^+$  concentrations. This was suggested to be due to reduced control of loading of  $\text{Na}^+$  to the xylem. Identification of the molecular basis of this mutation is eagerly awaited.

*Retrieval from the xylem.* Another strategy to reduce  $\text{Na}^+$  accumulation in the shoot may be the retrieval of  $\text{Na}^+$  that has entered the xylem before it reaches the bulk of the shoot.

This could occur in either: (a) the mature root (e.g. Shone *et al.*, 1969; Yeo *et al.*, 1977b; Kramer, 1983); (b) the mesocotyl (Johansen and Cheeseman, 1983; Drew and Läuchli, 1987); (c) the base of the shoot (e.g. Matsushita and Matoh, 1992); or (d) the mature extended shoot (e.g. Blom-Zandstra *et al.*, 1998).

The mechanisms of Na<sup>+</sup> removal from xylem sap are unknown. A likely pathway is via an Na<sup>+</sup>-permeable channel, such as an inwardly rectifying channel described in the xylem parenchyma cells of barley (Wegner and Raschke, 1994). However, Lacan and Durand (1996) proposed that in high Na<sup>+</sup>, an Na<sup>+</sup>/H<sup>+</sup> antiporter worked in reverse, to pump Na<sup>+</sup> into the cytosol. However, given an approximate 100-fold difference in H<sup>+</sup> activity between the cytosol and apoplast, if the exchange is electroneutral, the proposed antiport would be very unlikely to be thermodynamically possible in any physiologically likely situation (as apoplastic Na<sup>+</sup> would need to be 100-fold higher than that found in the cytosol—which is probably 10 mM or so).

Nevertheless, it is possible that an 'Na<sup>+</sup>/H<sup>+</sup> antiporter' does not always act *in vivo* as an electroneutral 1Na<sup>+</sup>/1H<sup>+</sup> antiporter. For example, it may move more than one Na<sup>+</sup> ion in at a time, or even act as an Na<sup>+</sup> uniporter. Shi *et al.* (2002) propose that SOS1 could act as an Na<sup>+</sup>-scavenging mechanism at the root xylem–symplast interface in high NaCl conditions (100 mM), suggesting that in these conditions either xylem Na<sup>+</sup> was extremely concentrated (although it was measured as low millimolar), or that the stoichiometry of Na<sup>+</sup>/H<sup>+</sup> exchange via SOS1 is not fixed (Shi *et al.*, 2002). Variable activity of solute-coupled transporters has already been proposed in plants, with the wheat K<sup>+</sup>/Na<sup>+</sup> symporter HKT1 acting as an Na<sup>+</sup> uniporter at elevated levels of Na<sup>+</sup> (Rubio *et al.*, 1995; see 'Ca<sup>2+</sup>-insensitive pathways', above).

The basis for the proposal by Shi *et al.* (2002) for this switch in activity of SOS1 was the observation that *sos1* mutants have less Na<sup>+</sup> in their shoot at modest salinities (due to loss of the pumping of Na<sup>+</sup> into the xylem), but they have higher shoot Na<sup>+</sup> concentrations at higher salinity (100 mM). However, in our hands, vigour of seedlings of even wild type *Arabidopsis* is severely reduced by this concentration of Na<sup>+</sup>, and the elevated shoot Na<sup>+</sup> in these conditions may simply indicate a general collapse of plant functions rather than a specific reversal of one transporter.

Interestingly, anoxic treatment of maize roots tended to increase transfer of Na<sup>+</sup> from roots to shoots at modest salinities (11 mM), but had a small inhibitory effect at higher salinity (40 mM) (Drew and Dikumwin, 1985). This is consistent with an active Na<sup>+</sup> retrieval mechanism from the xylem operating at lower Na<sup>+</sup> concentrations, but a passive removal from the xylem may occur at higher salinity, although effects of membrane depolarization on passive Na<sup>+</sup> influx and xylem loading must also be borne in mind.

The role of transfer cells is also very relevant to this discussion (Kramer, 1983). In the root, transfer cell-like protuberances can be seen near the xylem in the proximal part of the root only where xylem annular thickenings are not present (Läuchli *et al.*, 1974; Kramer *et al.*, 1977; Yeo *et al.*, 1977a). Commonly associated with these sites of increased membrane area is a higher density of mitochondria (Wooding, 1969; Läuchli *et al.*, 1974; Yeo *et al.*,

1977a). Crucially, their abundance well back (25 cm away) from the root tip increases noticeably upon subjection of maize (Yeo *et al.*, 1977a) and bean (Kramer *et al.*, 1977) plants to moderate salinity. It seems possible that they increase in order to remove more Na<sup>+</sup> back out of the xylem.

However, if retrieval from the xylem is an important adaptation to salinity, where does the Na<sup>+</sup> go? Some can be accumulated in vacuolate cells, such as seen in mature roots (Yeo *et al.*, 1977b), at the base of stems (Johansen and Cheeseman, 1983; Drew and Läuchli, 1987) and along stems in plants with elongated stems (Shone *et al.*, 1969; Wolf *et al.*, 1991; Blom-Zandstra *et al.*, 1998). Na<sup>+</sup> concentrations in xylem sap decreased up the elongated stem of adult barley plants growing in 100 mM NaCl, from 10 mM near the base to 2 mM near leaf 8 (Fig. 3A in Wolf *et al.*, 1991). This decrease, of approximately 1 mM per internode, equated to the removal of about 3 μmol d<sup>-1</sup> per internode, which was accounted for as an increase by that amount in the internodal tissue (Figs 1 and 6, Wolf *et al.*, 1991). This increase in Na<sup>+</sup> concentration led, by day 45, to total tissue concentrations of up to 350 μmol g<sup>-1</sup> f. wt (Fig. 1, Wolf *et al.*, 1991). It would appear that protection of the growing tissue could be significantly facilitated by removal of Na<sup>+</sup> from the transpiration stream and accumulation of very high concentrations in internodal tissues. This process is feasible owing to the short life of the barley plant and its rapid growth rate, which helps keep new tissue 'ahead' of the advancing accumulation of Na<sup>+</sup>.

## 2. Recirculation in the phloem

Recirculation of Na<sup>+</sup> back to the roots by the phloem to levels that significantly influence the total extent of leaf Na<sup>+</sup> accumulation has been reported in several species, including lupin (Munns *et al.*, 1988), *Trifolium alexandrinum* (Winter, 1982), sweet pepper (Blom-Zandstra *et al.*, 1998) and maize (Lohaus *et al.*, 2000). Furthermore, two published comparative studies indicate that the extent of this recirculation is related to the tolerance of plants to salinity. Perez-Alfocea *et al.* (2000) found greater recirculation of Na<sup>+</sup> in the phloem of a salt-tolerant wild species of tomato, *Lycopersicon pennellii*, compared with that in domesticated tomato; and Matsushita and Matoh (1991) found much higher retrieval of <sup>22</sup>Na<sup>+</sup> from the shoot of salt-tolerant *Phragmites communis* than from rice. These data suggest that contrary to the 'textbook view', movements of Na<sup>+</sup> out of leaves in the phloem may be significant, and certainly warrant further investigation regarding their mechanism and control. However, the conditions under which retranslocation occurs, and the direction of retranslocation, are critical. For instance, sweet pepper grown in a split-root system did not retranslocate <sup>22</sup>Na<sup>+</sup> from shoot to roots, except when NaCl was removed from the root solution (Blom-Zandstra *et al.*, 1998). Similar considerations apply to leaf-feeding experiments, where often very small amounts of Na<sup>+</sup> are introduced to leaves under non-saline conditions, and the Na<sup>+</sup> may be in quite different, more mobile compartments than would be the case in saline conditions.

### 3. Compartmentation within the shoot

Protection of the young leaves has been proposed to be a crucial feature necessary for salt tolerance (e.g. Jeschke, 1984). This is most likely to be due to the paucity of vacuoles in these cell types (for the sequestration of Na<sup>+</sup>) and the sensitivity to Na<sup>+</sup> of active growth processes (notably protein synthesis). Low Na<sup>+</sup> is often observed in young leaves, and this has been attributed to both their low rates of transpiration and their relatively short existence (Munns, 1993). However, this does not preclude the additional possibility of preferential removal of Na<sup>+</sup> from the xylem and phloem that may be feeding these tissues.

It is plausible that Na<sup>+</sup> is preferentially removed from solutions moving towards organs that the plant needs to protect, and for such Na<sup>+</sup> to be either stored at the site of removal (discussed in 'Retrieval from the xylem', above) or directed towards 'sacrificial' parts of the shoot, such as older leaves. Such redirection of Na<sup>+</sup> could be done by movement down in the phloem and movement back up in the xylem, with the net effect of directing Na<sup>+</sup> towards a particular part of the shoot. Such extensive cycling of solutes in phloem and xylem has often been described for other solutes (e.g. Pate *et al.*, 1979), and there are some observations consistent with Na<sup>+</sup> being directed towards target leaves (Wolf *et al.*, 1991). Furthermore, selective movements of solutes within the phloem have been observed. For example, an autoradiogram in Marschner (1995, p. 95) shows movement of <sup>32</sup>P from a source leaf throughout a young bean plant, but <sup>22</sup>Na<sup>+</sup> moving out from the source leaf did not enter the younger regions of either the root or shoot. This is presumably due to the selective removal of Na<sup>+</sup> from the phloem before it reached the young parts of the plant. Thus, the components exist for such intra-organ compartmentation, although its significance in whole plant tolerance is not yet known.

Likewise, within a given leaf, preferential accumulation of Na<sup>+</sup> in epidermal cells has been described and attributed to increased activity of non-selective cation channels in the plasma membrane of epidermal cells (Karley *et al.*, 2000a, b). Intuitively, it might be expected that Na<sup>+</sup> accumulation in the more vacuolate, less metabolically active cells of the epidermis could be of adaptive value. High concentrations of Na<sup>+</sup> in bundle sheath cells suggest that this cell type may also be involved in attempts to minimize Na<sup>+</sup> accumulation in the crucial photosynthetic cells of the leaf mesophyll (Stelzer, 1981; Karley *et al.*, 2000b).

### 4. Salt glands

Many halophytes have salt glands of characteristic, although diverse, structure in their leaves. Their role is to remove salt that has reached the shoot onto the surface of the leaf, where it is separated from living cells by the waxy cuticle. Once removed, the salt can either crystallize in the sun or wash off in the rain. By pumping salt into a small volume of apoplast that is sealed off from the rest of the leaf apoplast, the resulting more negative osmotic potential causes water flow into this restricted space, causing a pressure build-up and subsequent bulk flow of solution

through a deliberate weakness between this apoplast and the leaf surface. As such, glands remove both salt and water from the leaf, and the loss of water restricts these plants to habitats where water is readily available, such as salt marshes.

Salt glands are found in a small number of evolutionarily distantly related plants, which have each evolved quite complex glands of distinct structures (Hill and Hill, 1976). This suggests that salt glands must be relatively easy to evolve. As such, their relatively low abundance suggests that there is a significant cost in their construction and/or operation.

Many grasses have small, bicellular glands that appear to secrete salt (Amarasinghe and Watson, 1989; McWhorter *et al.*, 1995). When several species within the subfamily Chloridoideae were compared, the salt-secreting activity of these glands was correlated with the salt-tolerance of the species (Marcum, 1999). Thus, conferring onto the leaf hairs of graminaceous crops the ability to secrete salt could be a valid strategy for increasing salinity tolerance. Notably, there is a wild relative of rice, *Porteresia coarctata*, that can grow in 25 % sea water, and has salt-secreting microhairs (Flowers *et al.*, 1990). One problem with this idea is the philosophically (rather than physiologically) based concern that although glands have evolved several times, they are not particularly widespread.

Halophytes living in drier habitats tend to contain 'salt hairs', two cells on the surface of the leaf, the outer of which accumulates salt and water, swells and eventually dies. Less water is lost when Na<sup>+</sup> is excreted from this cell type than is lost from secretory glands.

Another way of disposing of excess salt may be to lose salt from the hydathodes, which release water at times of low transpirational water loss, such as at night. Guttation is very common from leaves of cereals, and may be a pathway for loss of salts from cereals. Perhaps a salt-pumping capability could be introduced into the cells lining the hydathodes to increase salt loss by this route.

### 5. Control of transpiration—stomatal closure

Lack of an ability to close stomata in saline conditions has been proposed as an important reason for sensitivity to saline soils in some plants (Robinson *et al.*, 1997). For example, an ability to grow in saline conditions has been attributed to an ability to close stomata in the presence of leaf apoplastic Na<sup>+</sup> in a salt-tolerant species of aster, *Aster tripolium*, whereas salt-sensitivity was attributed to an inhibition of stomatal closure by Na<sup>+</sup> in *A. amellus* (and in other more extensively studied glycophytes; Robinson *et al.*, 1997). This ability to close guard cells in the halophytic aster was correlated with an ability to down-regulate inward-rectifying K<sup>+</sup> channel activity in response to a rise in cytosolic Na<sup>+</sup> (Véry *et al.*, 1998). This effect appeared to be mediated by a rise in cytosolic Ca<sup>2+</sup>. However, these data were obtained in leaf epidermal strips or guard cell protoplasts exposed to NaCl solutions, and are not easily reconciled with whole plant responses to soil Na<sup>+</sup>. In fact, both glycophytes and halophytes tend to show reduced stomatal conductance in high NaCl conditions (Bell, 1988;

Robinson *et al.*, 1997; James *et al.*, 2002). Given that the productivity of crops in saline soils is limited to a large extent by the low rate of gas exchange imposed by the drought component of NaCl stress, any further reduction in stomatal conductance and photosynthetic rate is unlikely to improve plant productivity (although it may increase survival).

### CONCLUSIONS

Tolerance to salinity involves processes in many different parts of the plant, and more than one of these processes can operate concurrently within a particular plant. These mechanisms can occur at a wide range of organizational levels, from the cellular (e.g. compartmentation of Na<sup>+</sup> within cells) to the whole plant (e.g. exclusion of Na<sup>+</sup> from the plant, and intraplant allocation of Na<sup>+</sup>). They can occur in all cells within the plant, or in specific cell types. This reflects adaptations at two major levels of organization: those that confer tolerance to cells, and those that contribute to tolerance not of cells per se, but of the whole plant.

Salt-tolerant cells can contribute to the salt-tolerance of plants, and constitutive changes in tonoplast transporters have been shown to increase salt tolerance. Nevertheless, we suggest that processes involving the management of Na<sup>+</sup> movements within the plant are just as critical to salt tolerance as cellular tolerance. Halophytes require both cellular and whole plant adaptations, and the persistent inability to regenerate vigorous salt-tolerant plants from salt-tolerant cells selected *in vitro* suggests that a rapidly growing salt-tolerant plant requires more than simply salt-tolerant cells.

The management of Na<sup>+</sup> movements within the plant requires specific cell types in specific locations within the plant catalysing transport in a coordinated manner. For example, to minimize Na<sup>+</sup> delivery to the shoot in the apoplastic compartment of the xylem, plasma membrane transport processes in the outer and inner halves of the root would need to be manipulated in opposite directions. To further understand complex, whole-plant adaptations to salinity, more information is required about cell-specific transport processes and the consequences of the manipulation of transporters and signalling elements in specific cell types.

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