Avenues for increasing salt tolerance of crops, and the role of physiologically based selection traits

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Abstract

Increased salt tolerance is needed for crops grown in areas at risk of salinisation. This requires new genetic sources of salt tolerance, and more efficient techniques for identifying salt-tolerant germplasm, so that new genes for tolerance can be introduced into crop cultivars. Screening a large number of genotypes for salt tolerance is not easy. Salt tolerance is achieved through the control of salt movement into and through the plant, and salt-specific effects on growth are seen only after long periods of time. Early effects on growth and metabolism are likely due to osmotic effects of the salt, that is to the salt in the soil solution. To avoid the necessity of growing plants for long periods of time to measure biomass or yield, practical selection techniques can be based on physiological traits. We illustrate this with current work on durum wheat, on selection for the trait of sodium exclusion. We have explored a wide range of genetic diversity, identified a new source of sodium exclusion, confirmed that the trait has a high heritability, checked for possible penalties associated with the trait, and are currently developing molecular markers. This illustrates the potential for marker-assisted selection based on sound physiological principles in producing salt-tolerant crop cultivars.

The problem

About 7% of the world's total land area is affected by salt, as is a similar percentage of its arable land (Ghassemi et al., 1995; Szabolcs, 1994). The area is still increasing as a result of irrigation or land clearing (FAOSTAT).

The major salinity problem in Australia is 'dryland salinity', i.e., it results from land clearing. It is due to rising water tables resulting from clearing the original native vegetation, which consisted largely of perennial species. Water tables rise because annual crops allow more rainwater to escape their root systems than the original perennial vegetation. In the Australian wheat belt, an average of about 30 mm of water is added to the groundwater each year (Dunin et al., 2001). This raises the water table by about half a metre each year,

carrying salts that have accumulated in the soil. When the water table eventually reaches the surface the water evaporates, leaving any salt behind. In Australia, this process may ultimately affect a very large proportion of the area that has been cleared for farming. Predictions by a recent national land and water resources audit (ANRA, 2001) indicate that by the year 2050, as many as 17 million ha will be salinised, or be at risk of salinisation. This area represents a third of Australia's agricultural land area.

Irrigation systems are particularly prone to salinisation, with about half the existing irrigation systems of the world now under the influence of salinisation or waterlogging, due to either low quality irrigation water, or to excessive leaching and subsequent rising water tables (Szabolcs, 1994). Irrigation schemes cover only 15% of the cultivated land of the world, but as irrigated land has at least twice the productivity of rainfed land, it may produce one third of the world's food.

The solution

Salinisation can be restricted by changed farm management practices. Irrigated agriculture can be sustained by better irrigation practices such as adoption of partial root zone drying methodology, and drip or micro-jet irrigation to optimise use of water. The spread of dryland salinity can be contained by reducing the amount of water passing beyond the roots. This can be done by re-introducing deep-rooted perennial plants, that continue to grow and use water during the seasons that do not support annual crop plants. This may restore the balance between rainfall and water use, thus preventing rising water tables and the movement of salt to the soil surface.

Farming systems can change to incorporate perennials in rotation with annual crops (phase farming), in mixed plantings (alley farming, intercropping), or in site-specific plantings (precision farming). Precision farming can identify areas giving consistently poor yield, and these can be excluded from cropping, as such areas are especially prone to 'leakage' (i.e., allowing rainwater to escape below the roots). In precision farming, areas of high production can also be identified, and these sites can be planted with cultivars of high vigour that use water effectively during the growing season, and consume most of the available soil water. Phase farming, in which several years of pasture are rotated with several years of crop, can make use of deep-rooted pasture plants to dry the deep subsoil, thereby creating a buffer zone to hold any water that escapes the crops. Trials in Australia have shown that the deep-rooted perennial lucerne (*Medicago alfalfa*) can lower the water table sufficiently to allow subsequent cropping (Ridley et al., 2001). Such practices will rely on plants that have a high degree of salt tolerance. Tolerance will be required for the 'de-watering' species, but also for the annual crops to follow, as salt will be left in the soil when the water table is lowered. Salt tolerance in crops will also allow the more effective use of poor quality irrigation water.

Diversity in salt tolerance between species

Salt tolerance is usually assessed as the percent biomass production in saline versus control conditions over a prolonged period of time. Figure 1 shows the effect of salinity on lupin (one of the most salt-sensitive crop species), barley (one of the most tolerant), as well

as two halophytes that are useful forage in salt-affected soils. The data shown in the figure are for plant dry weight after a period of about a month in a range of salinities. The data are from experiments in which the salinity increased after plants were established in non-saline conditions, not for experiments when salt was added at germination. The figure indicates that in a field where the salinity rises to 100 mM NaCl (about 10 dS m^{-1}), lupins, and in fact most legume species, will die before maturity, while crops such as wheat and barley will produce a reduced yield. Even barley dies at salt concentrations higher than 250 mM NaCl (about 25 dS m^{-1}, or 50% seawater). Only halophytes will cope with soils where the watertable has brought salt to the surface, as the water in the topsoil will contain salts at concentrations higher than seawater.

The effects of salinity on barley and lupin probably span the extremes of salt tolerance of crops. Wheat (*Triticum aestivum*) is usually considered less tolerant than barley, but there is such difference between genotypes that it is difficult to make a categorical statement. Bean (*Phaseolus vulgaris*) is one of the most salt-sensitive species, but for this species, like so many, the supply of additional Ca^{2+} is crucial for the salt tolerance (Lahaye and Epstein, 1971), and again it is difficult to generalise. Rice (*Oryza sativa*) is regarded as one of the more salt-sensitive crops, which is certainly true when grain yield is considered (Khatun et al., 1995; Maas and Hoffman, 1977). However, vegetative growth of some rice cultivars can be surprisingly tolerant of salinity, at least when adequate Ca^{2+} is supplied (Muhammed et al., 1987).

Another criterion of salt tolerance of crops is their yield in saline versus non-saline conditions. A survey of salt tolerance of crops, vegetables and fruit trees has been published by Maas and Hoffman (1977), and updated by Francois and Maas (1994). They show for each species a threshold salinity below which there is no reduction in yield, and then a regression for the reduction in yield with increasing salinity. The data in some cases are for a single cultivar of the species, or a limited number of cultivars at a single site, so they are not necessarily representative of the species. However, the data are useful in that they show the wide range of tolerance across species, and also show that yield has a different pattern of response than does vegetative biomass. Yield always shows a threshold in response to a range of salinities (Maas and Hoffman, 1977), but with young plants a threshold in growth is rarely seen. With plants exposed to salinity at an early stage of seedling development there are linear reductions in
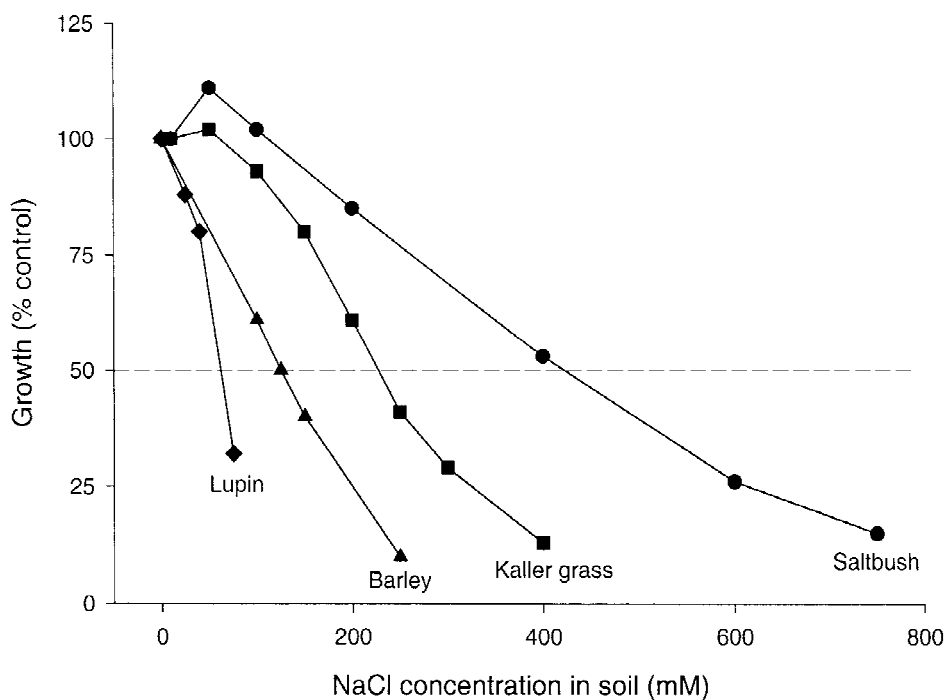
Figure 1. Growth of four diverse plant species to a range of salinity levels. The saltbush species is *Atriplex amnicola*, a halophyte of Western Australia (data from Aslam et al., 1986). Kallar grass, *Diplachne* (syn.*Leptochloa*) *fusca*, is widespread in many continents including Australia (data from B. Myers and D. West, pers. commun.). Barley (*Hordeum vulgare*) is one of the most salt-tolerant crops (data from Greenway, 1962; Rawson et al., 1988) and white lupin (*Lupinus albus*) is one of the most sensitive (data from Jeschke et al., 1986).

both leaf area expansion and total plant biomass with increasing salinity, as shown in Figure 1.

There is probably a great diversity in salt tolerance within species that has not been fully explored. One reason for this is the difficulty of measuring the tolerance of salinity as distinct from the tolerance of water or osmotic stress, and the difficulty of screening large numbers of individuals for small, repeatable and quantifiable differences in biomass production, let alone yield.

How to screen for small differences in salt tolerance within species

Differences in salt tolerance between closely related species are difficult to quantify, as the growth reduction depends so much on the period of time over which the plants have grown in saline conditions. Salinity lowers the water potential of the roots, and this quickly causes reductions in growth rate, along with a suite of metabolic changes identical to those caused by water stress (reviewed by Munns, 2002). Later, there

may be salt-specific effects that impact on growth or senescence.

The first few days or weeks in salinity may reveal no differences in growth response between species that have quite different reputations for salt tolerance. For example, durum wheat, *Triticum turgidum* ssp. *durum* is much more salt-sensitive than bread wheat, *Triticum aestivum* (Francois et al., 1986; Rawson et al., 1988), yet over short periods of time in salinity we found no differences between durum and bread wheat cultivars (Munns et al., 1995). In a comparison between 20 cultivars of wheat, barley and triticale we found no significant differences between the leaf elongation rate in the first 10 days of salinisation of any cultivar, including that of the one that ultimately turned out to be the most sensitive (a durum wheat) and the one (a barley) that turned out to be the most tolerant (Rawson et al., 1988). Similar results have been obtained recently with other wheat lines that have a reputation of differing in salt tolerance. Four weeks of growth at 150 m*M* NaCl was insufficient time for difference in salt tolerance between genotypes to show up (Rivelli et al., 2002), including bread and durum wheat cultivars that were known to differ in salt tolerances in the field.
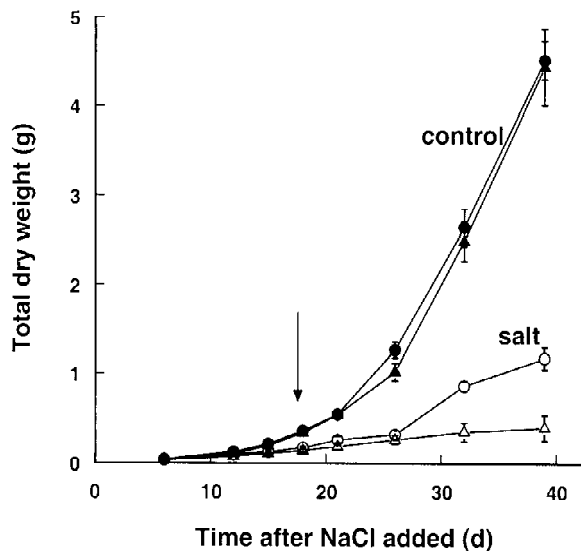
Figure 2. Two accessions of the diploid wheat progenitor *Ae. tauschii* grown in supported hydroponics in control solution (closed symbols) and in 150 mM NaCl with supplemental Ca^{2+} (open symbols). Circles denote the tolerant accession, triangles the sensitive one. The arrow marks the time at which symptoms of salt injury could be seen on the sensitive accession; at that time the proportion of dead leaves was 10% for the sensitive and 1% for the tolerant accession (Munns et al., 1995). A similar result is given in Fortmeier and Schubert (1995).

These data are consistent with the concept of a two-phase growth response to salinity (Munns, 1993). The first phase of growth reduction is quickly apparent, and is due to the salt outside the roots. It can be called a water stress or osmotic phase, for which there is surprisingly little genotypic difference. Then there is a second phase of growth reduction, which takes time to develop, and is associated with advanced senescence of older leaves. This presumably results from internal injury due to salts accumulating in these transpiring leaves to excessive levels. If excessive amounts of salt enter a plant, salt will eventually rise to toxic levels in the older transpiring leaves, causing premature senescence and reducing the photosynthetic capacity of the plant to a level that cannot sustain further growth (Munns, 1993). The cause of the injury is probably due to the salt load exceeding the ability of the cells to compartmentalise salts in the vacuole. Salts then would rapidly build up in the cytoplasm and inhibit enzyme activity. Alternatively, they might build up in the cell walls and dehydrate the cell. Evidence for ions accumulating to high concentrations in the apoplast of leaves has been found in rice (Flowers et al., 1991), but not maize (Mühling and Läuchli, 2002).

A two-phase growth response has been shown clearly for maize and wheat cultivars. Two maize cultivars with 2-fold differences in rates of Na^+ accumulation in leaves had the same growth reduction for 15 days in 80 mM NaCl (Cramer et al., 1994). Another two maize cultivars, again with 2-fold differences in Na^+ accumulation, had the same growth reduction for 4 weeks in 100 mM NaCl, and it was not until 8 weeks that a growth difference was clearly seen (Fortmeier and Schubert, 1995). Similar results were found in wheat (Munns et al., 1995). Two closely-related wheat genotypes that differed in rates of Na^+ accumulation had the same growth reduction for 4 weeks in 150 mM NaCl, and it was not until after 4 weeks that a growth difference between the genotypes was clearly seen (Figure 2). However, within 2 weeks dead leaves became visible on the more sensitive genotype (Figure 2), and the rate of leaf death was clearly greater on the sensitive than the tolerant genotype. Once the number of dead leaves increased above about 20% of the total, plant growth slowed down and many individuals started to die (Munns et al., 1995).

With rice, also, a clear distinction has been made between the initial effects of salinity, from which recovery is possible, and the long-term effects that result from the accumulation of salt within expanded leaves (Yeo et al., 1991).

These observations illustrate the principle that the initial growth reduction is due to the osmotic effect of the salt outside the roots, and that what distinguishes a salt-sensitive plant from a more tolerant one is the inability to prevent salt from reaching toxic levels in the transpiring leaves, which takes some time.

The length of time required before growth differences between genotypes can be seen depends on the salinity and the degree of salt tolerance of the species. The second phase will start earlier in plants that are poor excluders of Na^+, such as lupins or beans, and when salinities are higher. It will also start earlier when root temperatures are higher. For plants such as rice that are grown at high temperatures, 10–15 days in salinity is sufficient to generate differences in biomass between genotypes that correlate well with differences in yield (Aslam et al., 1993).

The labour and space demands of these long experiments makes this impractical for screening large numbers of genotypes, or selecting salt-tolerant progeny. This means that our knowledge of physiological mechanisms should be used to identify traits that can be employed for rapid and cost-effective selection techniques.
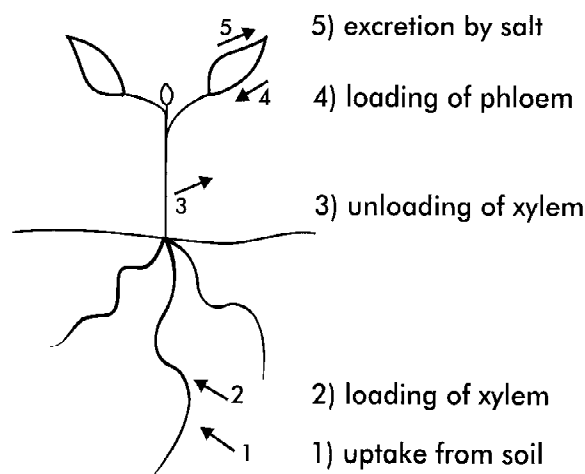
Figure 3. Control points at which salt transport is regulated. These are: (1) selectivity of uptake from the soil solution; (2) loading of the xylem; (3) removal of salt from the xylem in the upper part of the plant; (4) loading of the phloem; and (5) excretion through salt glands or bladders. For a salt tolerant plant growing for some time in a soil solution of 100 mM NaCl, the root concentrations of Na$^+$ and Cl$^-$ are typically about 50 mM, the xylem concentration about 5 mM, and the concentration in the oldest leaf as high as 500 mM. See text for explanation.

Mechanisms of salt tolerance

Mechanisms of salt tolerance take place at three levels of organisation: whole plant, cellular and molecular.

Control at the whole plant level

Physiological mechanisms conferring exclusion that operate at the cellular and whole plant level have been described in previous reviews (Greenway and Munns, 1980; Läuchli, 1984; Munns et al., 1983; Pitman, 1984; Storey and Walker, 1999), and with particular reference to selectivity for K$^+$ over Na$^+$ (Jeschke, 1984; Jeschke and Hartung, 2000). Salt tolerance depends on the ability of the plant to control the transport of salt at five sites (Figure 3), as summarised below:

1. Selectivity of uptake by root cells. It is still unclear which cell types control the selectivity of ions from the soil solution. The initial uptake of Na$^+$ and Cl$^-$ could occur at the epidermis, at the exodermis, or if soil solution flows apoplastically across the root cortex, it would occur at the endodermis.
2. Loading of the xylem. There is evidence for a preferential loading of K$^+$ rather than Na$^+$ by the cells of the stele.
3. Removal of salt from the xylem in the upper part of the roots, the stem, petiole or leaf sheaths. In many species, Na$^+$ is retained in the upper part of the root system and in the lower part of the shoot, indicating an exchange of K$^+$ for Na$^+$ by the cells in the stele of the roots or in the vascular bundles in stems and petioles.
4. Loading of the phloem. There is little retranslocation of Na$^+$ or Cl$^-$ in the phloem, particularly in the more tolerant species. This ensures that salt is not exported to growing tissues of the shoot.
5. Excretion through salt glands or bladders. Only halophytes have these specialised cell types.

All halophytes have well-developed mechanisms to control the uptake, transport and excretion of salt. Glycophytes rely on the first three mechanisms, and exhibit these mechanisms to various degrees. Genetic variation within a given species, or between closely related species, has in most cases been identified as due to different degrees of control of salt uptake by roots, or in loading of the xylem. Genetic variation in Na$^+$ loading of the xylem appears to explain differences in Na$^+$ accumulation and hence salt tolerance between *Triticum* species (Gorham et al., 1990). Exclusion is particularly important for perennials, the leaves of which may live for a year or more; there is greater need to regulate the incoming salt load over a much longer period of time than for annual species whose leaves may live for only 1 month.

There are contributory features that function to maintain low rates of salt accumulation in leaves. High shoot:root ratios and high intrinsic growth rates (Pitman, 1984), and absence of an apoplastic pathway in roots (Garcia et al., 1997) all will serve to reduce the rate at which salt enters the transpiration stream and accumulates in the shoot.

Control at the organelle level: ion compartmentation

There is no evidence of adaptations in enzymes to the presence of salt (reviewed by Munns et al., 1983), so mechanisms for salt tolerance at the cellular level involve keeping the salt out of the cytoplasm, and sequestering it in the vacuole of the cell. That this occurs in most species is indicated by the high concentrations found in leaves that are still functioning normally, concentrations well over 200 mM, yet we know that these same concentrations will completely repress enzyme activity *in vitro* (Munns et al., 1983). Generally, Na$^+$ starts to inhibit most enzymes at a concentration above 100 mM. The concentration at which Cl$^-$ becomes toxic is even less well defined, but is probably in the same range as that for Na$^+$. If Na$^+$ and Cl$^-$ are se-

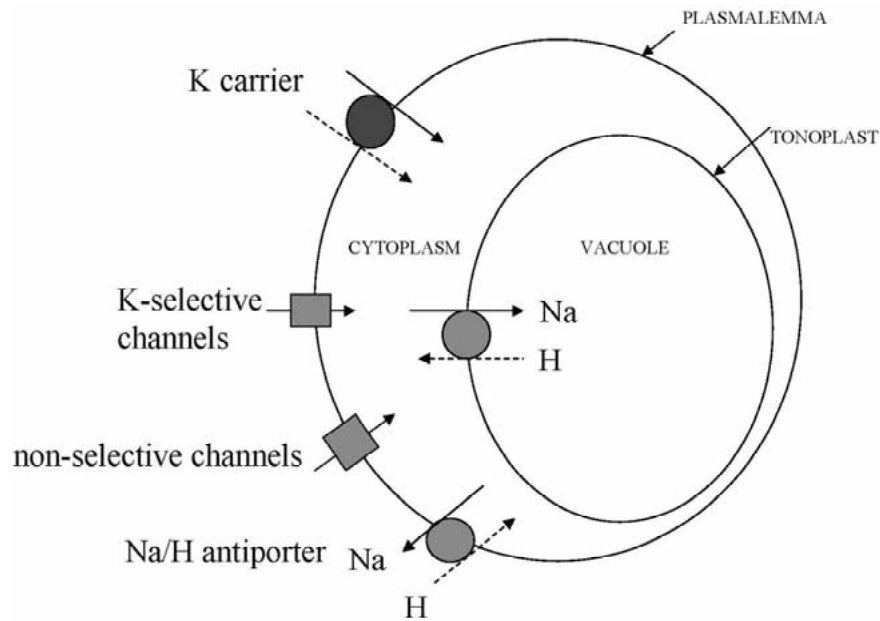*Figure 4.* Mechanisms of Na$^+$ transport in higher plants. Regulation of Na$^+$ uptake across the plasmalemma would come from restricted uptake by selective cation transporters and channels, coupled with efflux by the antiporter. The antiporter on the tonoplast sequesters Na$^+$ in the vacuole. Adapted from Amtmann and Sanders (1999).

questered in the vacuole of the cell, K$^+$ and organic solutes should accumulate in the cytoplasm and organelles to balance the osmotic pressure of the ions in the vacuole. The organic solutes that accumulate most commonly under salinity are proline and glycine betaine, although other molecules can accumulate to lesser degrees (summarised in Hasegawa et al., 2000, their Figure 1).

Control at the molecular level: ion transporters

The ion channels and transporters that regulate the net movement of salt across cell membranes have been recently reviewed (Amtmann and Sanders, 1999; Blumwald, 2000; Schachtman and Liu, 1999; Tyerman and Skerritt, 1999). The mechanisms that control Na$^+$ transport are summarised in Figure 4. There is no specific Na$^+$ transporter, Na$^+$ entry being gained by competition with other cations, in particular K$^+$. Na$^+$ could enter the cell through high affinity K$^+$ carriers or through low affinity channels called non-selective cation channels that are strongly influenced by Ca^{2+}. These cation channels could allow entry of large amounts of Na$^+$ from a highly saline soil if not adequately regulated (Amtmann and Sanders, 1999). Na$^+$ can be effluxed from the cytoplasm through Na$^+$/H$^+$ antiporters, driven by the pH gradient across

the plasmalemma (Blumwald, 2000). These transport processes all work together to control the rate of net uptake of Na$^+$ by a cell (Figure 4). Intracellular compartmentation is by a vacuolar Na$^+$/H$^+$ antiporter, driven by a pH gradient across the tonoplast (Blumwald et al., 2000). The transporters that maintain low Na$^+$ concentrations in organelles such as chloroplasts and mitochondria are not known. In some species, Cl$^-$ transport is associated with salt tolerance. Mechanisms that control Cl$^-$ movement across membranes have been comprehensively reviewed by White and Broadley (2001).

Strategies for increasing salt tolerance: and the importance of traits

There are two main avenues for improving salt tolerance of a given crop or cultivated species. These are (1) searching amongst natural diversity within the species, or closely related and inter-fertile species, and (2) genetic engineering. With both avenues, backcrossing into cultivars or advanced breeding lines will be required. This requires precise screening of progeny, using either a quantitative trait or a molecular marker for that trait. Screening for a trait associated with a specific mechanism is preferable to screening for salt tolerance itself, as measuring the effect of salt

on biomass or yield of a large number of lines is not feasible. As mentioned above, plants need to be grown for lengthy periods of time, and controls need to be included, as the source of the salt tolerance could come from a parent or transformant that is taller or shorter or has a different growth rate than the cultivar into which the germplasm or gene is being introduced. In the field, the major drawback is the heterogenous nature of salinity within paddocks and between sites.

Traits for salt tolerance that have been used to screen germplasm collections have included rates of Na^+ or Cl^- accumulation in leaves, degree of leaf injury, seedling root length, and germination percentage. The most successful relate to rates of Na^+ or Cl^- accumulation in leaves, measured as the increase in salt in a given leaf over a fixed period of time. Sodium accumulation in leaves has been shown to relate to salt tolerance in genotypes of rice (Yeo and Flowers, 1986) and diploid wheat, *Aegilops tauschii* (Schachtman et al., 1991). Salt tolerance at germination is easy to measure, but little or no relation between salt tolerance at germination and that of the seedling or adult plant has been found in any species examined, including barley (e.g., Mano and Takeda 1997), bread wheat (Kingsbury and Epstein, 1984), and durum wheat (Almansouri et al., 2001). In the approach described below, we use the rate of Na^+ accumulation in a given leaf as a non-destructive and accurate quantitative trait.

Molecular markers for these traits can provide an efficient selection technique in breeding programs. Some success has been reported for combining physiological traits in rice (IRRI, 1997). Molecular markers would be particularly useful for pyramiding different traits for salt tolerance (Flowers et al., 2000; Yeo and Flowers, 1986), and additionally for incorporating characters associated with other accompanying stresses, such as drought or waterlogging. We are therefore attempting to identify molecular markers for salt tolerance in wheat.

Physiology and genetics of sodium exclusion

Our current work is focussed on improving the salt tolerance of durum wheat (*Triticum turgidum* ssp. *durum*). Cultivated durum wheat is more salt-sensitive than bread wheat (*Triticum aestivum*), a feature that restricts its expansion into areas with sodic or saline soils. The trait that we have targeted is low rates of Na^+ uptake. Screening for this by a non-destructive

method (Na^+ concentration in a given leaf 10 days after emergence, in plants at 150 mM NaCl) has allowed us to identify novel sources of Na^+ exclusion in ancient landraces of durum wheat (Munns et al., 2000).

In wheat, salt tolerance is associated with low rates of transport of Na^+ to shoots with high selectivity for K^+ over Na^+; there is little genotypic variation in rates of Cl^- transport (Gorham, 1990). Bread wheat cultivars (hexaploid, AABBDD genomes) have a low rate of Na^+ accumulation and enhanced K^+/Na^+ discrimination, a character located on the long arm of chromosome 4D (Gorham et al., 1987). This character is controlled by a single locus (*Kna1*) and has been linked to molecular markers on the distal third of chromosome 4DL (Dubcovsky et al., 1996). The gene or genes associated with this locus have not been identified. Durum wheat cultivars (tetraploid, AABB genomes) have high rates of Na^+ accumulation and poor K^+/Na^+ discrimination (Gorham et al., 1987), and are less salt-tolerant than bread wheat. One approach to improve the salt tolerance of durum wheat has been to create novel germplasm with low accumulation of Na^+ and enhanced K^+/Na^+ discrimination by homologous recombination with chromosome 4D (Dvorak et al., 1994). This, however, brings in unwanted genetic material on the translocated chromosome segment, which cannot be eliminated. Another approach is to search for natural genetic diversity on the A or B genomes, and this is the approach we have taken.

(a) Genetic variation in Na^+ exclusion

In order to introduce salt tolerance into current durum wheat from sources other than the D genome, we searched for genetic variation in salt tolerance across a wide range of durum-related tetraploids representing five *Triticum turgidum* sub-species (*durum, carthlicum, turgidum, turanicum, polonicum*). Selections were screened for low Na^+ uptake and its associated enhanced K^+/Na^+ discrimination. Wide genetic variation was found (Munns et al., 2000). Low Na^+ accumulation (and high K^+/Na^+ discrimination) of similar magnitude to that of bread wheat was found in a landrace from the sub-species *durum* (Munns et al., 2000). Figure 5 illustrates the range of genetic variation in Na^+ uptake that exists in the *Triticum* genus. The low Na^+ values for the bread wheat 'Janz' are typical of bread wheat cultivars, and the higher Na^+ values for the durum wheats 'Tamaroi' and 'Wollaroi'
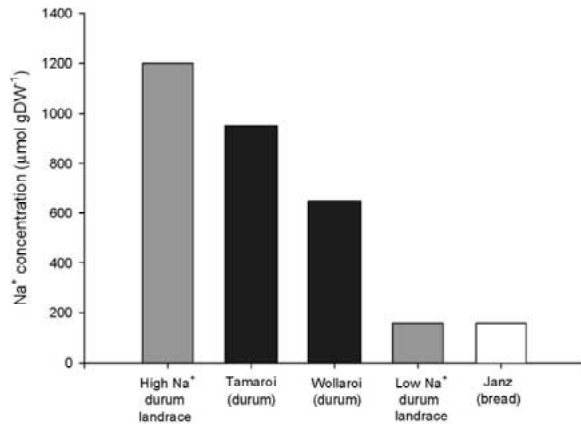
Figure 5. Sodium accumulation over 10 days in leaves of wheat genotypes. Plants were grown in supported hydroponics in 150 m*M* NaCl with half-strength Hoagland's solution and supplemental Ca^{2+}. Shown are the durum landraces with the highest and lowest Na^+ accumulation of the tetraploids screened by Munns et al. (2000), two current durum cultivars (Tamaroi and Wollaroi) and a represtative bread wheat (Janz).

are typical of current durum wheat cultivars. Most landraces examined had Na^+ values as high or higher than the cultivars, but a few had very low values. The landrace with the lowest rate of Na^+ accumulation is shown in Figure 5, and this trait is being incorporated into the durum cultivars through a backcrossing program.

(b) Inheritance of Na^+ exclusion

The low-Na^+ durum landrace (Figure 5) was crossed with the durum cultivar Tamaroi. The phenotypes of the F_1, F_2 individuals and $F_{2:3}$ families from this cross were determined by measuring the Na^+ accumulation in leaf 3 at 10 days after emergence, in plants grown at 150 m*M* NaCl. The F_1 progeny were intermediate between the parents in Na^+ accumulation, but about 10% of the F_2 progeny had Na^+ levels as low as the low-Na^+ parent. The distribution of Na^+ accumulation in individuals in the F_2 population and the $F_{2:3}$ families indicated that more than one gene was involved, most likely two or three genes of major effect (data not shown). Inheritance of this trait was assessed by regressing a selection of the fifteen highest and lowest $F_{2:3}$ family means with the corresponding single F_2 plant values. This showed that the trait was highly heritable with a high narrow sense heritability of 0.79 (±0.07) and therefore viable for use in a breeding program.

(c) Does Na^+ exclusion carry a penalty for water relations?

Four wheat genotypes with contrasting rates of Na^+ accumulation were selected to see if Na^+ exclusion resulted in poorer water relations during exposure to saline conditions. One genotype was the low-Na^+ landrace used in the durum improvement program described above. Another was the bread wheat 'Janz' with similarly low rate of Na^+ uptake. A third was the durum wheat 'Tamaroi' with high rates of Na^+ uptake while the fourth was a durum landrace with extremely high rates of Na^+ uptake. The Na^+ levels of these four genotypes are shown in Figure 5. Plants were grown in supported hydroponics, with and without 150 m*M* NaCl, and sampled for water relations, biomass, and ion accumulation over time. The results, as described by Rivelli et al. (2002), showed that there was little difference between genotypes in the effect of salinity on water relations, as indicated by their water potential, estimated turgor, and relative water content. Osmotic adjustment occurred in all genotypes, with one of the low-Na^+ genotypes having the greatest osmotic adjustment, and one of the high-Na^+ genotypes having the lowest osmotic adjustment. In the low-Na^+ genotypes, osmotic adjustment was enabled in part by the higher K^+, as Na^+ exclusion was always associated with maintenance of higher K^+ levels. Other solutes, such as Cl^- and organic solutes, also played a part (Rivelli et al., 2002). Over the 4-week period of this experiment, there was no significant difference between genotypes in the effect of salinity on growth. These data indicate that selecting lines with low Na^+ accumulation for the purpose of improving salt tolerance is unlikely to introduce adverse effects on plant–water relations or growth.

The reason why Na^+ exclusion might not impose a limitation to osmotic adjustment is that it is generally associated with K^+ accumulation. We asked the question: would Na^+ exclusion carry a penalty for water relations in soils with low K^+?

(d) Does low K^+ supply affect performance of low-Na^+ lines?

To answer this question, four genotypes with contrasting rates of Na^+ accumulation were selected. These were basically the same as the four genotypes used in the water relations experiment described above, differing only that the durum cultivar was 'Wollaroi' (see Figure 5). Plants were grown for 2 weeks at two K^+ levels, one representing an enriched soil, 3.3 m*M*,

the same as half-strength Hoagland's nutrient solution, the other representing a low-K^+ soil, 0.5 mM. There were three salinity levels: 1, 100 and 150 mM NaCl, with supplemental Ca^{2+} to overcome the salt effect on Ca^{2+} activity of the external solution.

As expected, the low K^+ supply reduced K^+ uptake and increased Na^+ uptake in all genotypes, but to different degrees depending on the ion, the salinity level and the genotype (data not shown). At 150 mM NaCl, the increased uptake of Na^+ entirely compensated for the decreased K^+ uptake, so that the sum of ($K^+ + Na^+$) was not significantly affected in any genotype by the low K^+ supply (Table 1). At 100 mM NaCl, the decreased K^+ uptake was not balanced by the increased uptake of Na^+ in two of the four genotypes, so there was a small but significant decrease in the sum of ($K^+ + Na^+$) in two genotypes. Only one of these was a low-Na^+ genotype. At 1 mM NaCl the sum of ($K^+ + Na^+$) was significantly reduced in all genotypes, as the decrease in K^+ uptake was greater than the increase in Na^+ uptake (Table 1). The growth rate was not affected by the low K^+ treatment at any of the three salinity levels over this experimental period of 2 weeks.

The experiment therefore showed that there were no effects of K^+ supply on the accumulation of Na^+ or K^+ in either the shoot (Table 1) or the root (data not shown) that would restrict the osmotic adjustment of the low-Na^+ genotypes in saline soil. Cl^- accumulation was not affected by the low K^+ supply (data not shown). Curiously, Ca^{2+} uptake was enhanced by low K^+ supply, by about one third, at all salinities and in all genotypes. Thus the higher external K^+ competed with both Na^+ and Ca^{2+} for uptake.

In summary, we identified a new source of Na^+ exclusion that can be incorporated into modern durum cultivars with no growth penalty. Isogenic lines with high versus low Na^+ accumulation are being developed, and will provide germplasm that can be evaluated in the field to test the concept that low Na^+ accumulation increases biomass production and yield of durum wheat in saline soil.

Molecular markers for physiological traits

The development of molecular markers for physiological traits has made significant headway in recent years with the advancement of new technologies. Consequently, the use of molecular markers in breeding programs is increasing rapidly as they have been shown to greatly improve the efficiency of the breeding programs. Although the application of molecular markers is relatively straightforward, the development of robust markers that are reliable across a wide range of backgrounds can be quite difficult, and is entirely dependent on an accurate phenotype screen. Understanding the physiology of sodium uptake is critical to the development of a reliable and accurate phenotype test, and thereby to the identification of QTLs and molecular markers.

QTLs (Quantitative Trait Loci) for salt tolerance have been described in several cereal species, including rice (Flowers et al., 2000; Koyama et al., 2001) barley (Ellis et al., 1997; Mano and Takeda, 1997), and bread wheat (Semikhodskii et al., 1997). However, these studies have not yet yielded robust markers that can be used across a range of germplasm, significant associations between the trait and the marker being confined to the populations in which they were derived. The success of these studies could be limited by the small amount of genetic diversity present within modern cultivars, and the use of parental lines with small differences in the traits. Our approach has been to seek a wider genetic diversity than exists in modern populations. This is possible with tetraploid and hexaploid wheats, as the progenitors of modern durum and bread wheats may have been derived from a limited germplasm base, and there may be genetic diversity present in original populations of the diploid ancestors that is not yet exploited.

Aegilops tauschii – *diploid wheat*

To develop markers for salt tolerance in bread wheat (AABBDD), we worked with *Ae. tauschii*, the diploid progenitor of bread wheat (DD). This species (syn. *Ae. squarrosa*, *Triticum tauschii*) was chosen because the D genome was shown to be responsible for the enhanced sodium exclusion of bread wheat as compared to durum wheat (Shah et al., 1987).

We searched a large collection of *Ae. tauschii* accessions for sodium exclusion (Schachtman et al., 1991). Accessions containing high and low sodium in the most recent fully expanded leaf (leaf 5) were selected and crossed to construct F_2 populations that could be mapped in search of markers linked to sodium exclusion. Three populations were created by crossing the selected accessions: CPI110835×CPI110791, CPI110664×AUS18905 and CPI110664×CPI110791. Progeny from the F_1 were grown in salt and Na^+ concentrations in leaf 5 were measured. Populations

Table 1. Effect of low K^+ supply (0.5 mM versus 3.3 mM K^+ in the soil solution) on the sum of (K^++Na^+) concentration in the whole shoot of wheat genotypes with low and high Na^+ uptake rates (details in text). Plants were grown for 2 weeks at three salinities: 1, 100 and 150 mM NaCl with supplemental Ca^{2+}(2, 8 and 10 mM Ca^{2+}, respectively). Asterisks denote significant differences at the $P = 0.05$ level. Genetic difference in leaf Na^+ concentrations in the four genotypes are shown in Figure 5 for 150 mM NaCl

| | $K^+ + Na^+$ (mmol g^{-1} DW) | | | | | |
| | 1 mM NaCl | | 100 mM NaCl | | 150 mM NaCl | |
External K^+ (mM)	3.3	0.5	3.3	0.5	3.3	0.5
Low-Na^+ durum landrace	1.21	1.00*	1.32	1.28	1.52	1.47
Low-Na^+ cv 'Janz'	1.26	0.96*	1.42	1.21*	1.52	1.48
High-Na^+ cv 'Wollaroi'	1.24	1.01*	1.70	1.54*	1.85	1.70
High-Na^+ durum landrace	1.22	0.97*	1.80	1.67	1.77	1.69

(74–96 individuals) were skewed to the low sodium parent, and there appeared to be transgressive segregation which produced individuals with lower Na^+ concentrations in leaf 5 than the parental accessions (Schachtman, 1991). These results indicated that multiple genes were associated with the sodium exclusion trait, although genes of major effect that confer sodium exclusion were evident. The population created from CPI110664×AUS18905 was used for mapping, and 43 out of 150 available RFLP probes were found to be polymorphic. A linkage map was then constructed with the 38 linked markers with five markers remaining unlinked. Regions of significant QTLs for Na^+ concentrations in leaf 5 were calculated along portions of the skeletal linkage groups for the *Ae. tauschii* genome using MAPMAKER QTL software. A single QTL (LOD score=2.1) was found on chromosome 4 between the markers that encode sequences for the loci controlling the early germination protein, germin, and the 7S globulin gene, 7SglobA. These markers are linked to the short arm of chromosome 4. At the time the analysis was completed markers on the long arm of the chromosome 4 were not available. With additional polymorphic markers the statistical significance of the QTL analysis could have been increased. In addition our analysis revealed that the sodium exclusion trait showed a low narrow sense heritability of 0.11, indicating that either the phenotyping was not sufficiently precise, or that there was a strong environmental influence (a large genotype by environment interaction). Phenotyping under a number of different environments would have clarified this. These results provided guidance for later work.

This mapping activity provided information on the dominance of the sodium exclusion trait and suggested that genetic improvement in sodium exclusion could be achieved by breeding. To test this, we determined whether the differences in salt tolerance and Na^+ accumulation in the different *Ae. tauschii* accessions would be expressed in synthetic hexaploid wheat, by crossing three of them with a common tetraploid wheat (Schachtman et al., 1992). The salt tolerance of the synthetic hexaploids was greater than the tetraploid parents primarily due to the maintenance of kernel weight. The synthetic hexaploids varied in salt tolerance according to the salt tolerance of the *Ae. tauschii* used in the cross, demonstrating that genes for salt tolerance from the diploid are expressed at the hexaploid level.

Durum wheat

The construction of a genetic map in durum wheat (AABB) and subsequent development of molecular markers for the trait of sodium exclusion is of particular interest to our group. As mentioned earlier, durum wheat lacks the D genome and the associated trait of sodium exclusion. We are attempting to find a source of this trait on the A or B genome, in old durum wheat landraces or related tetraploid species, and to introduce this trait into Australian durum cultivars. Improving the salt tolerance of Australian durum cultivars will enable the continued growth of these high-yielding and high-value crops in the Australian wheat belt, which is faced with rising water tables and the risk of dryland salinity (ANRA, 2001).

A population segregating for the low Na^+ uptake trait was developed from a cross between the low-Na^+ landrace and the cultivar 'Tamaroi' (see Figure 5), for which phenotypes of the F_2 individuals and $F_{2:3}$ families were determined by measuring the Na^+ accumulation in leaves 10 days after emergence, as described earlier. This population had shown a high level of heritability. DNA extracted from these individuals provided the material for genotypic analysis and the construction of the genetic map. Construction of a genetic linkage map based on AFLPs and microsatellites was initiated, to identify the chromosomal regions of major effect on Na^+ accumulation. Initially, 144 AFLP primer combinations were used to identify polymorphisms between the parental lines and bulked segregants of the 15 highest Na^+ uptake lines and 15 lowest Na^+ uptake lines. Twenty-three primer combinations were polymorphic, and with an average of five sites of polymorphism with each primer combination, approximately 100 polymorphic bands were available for scoring across the F_2 population. In addition, a screen of the parental lines using 112 microsatellites evenly distributed through the durum wheat genome identified a group of microsatellite markers that were polymorphic. Using a high stringency mapping approach ($P = 0.001$) several linkage groups were identified. Those with known map locations were identified and the marker densities in the identified regions were increased using RFLPs. Interval mapping using MapManager QTX version 13b revealed a QTL located on chromosome 2AL. This QTL showed significant association with the trait having a LOD score of 7.5. The analysis has shown that this locus accounts for half of the phenotypic variation of this trait. Other linkage groups did not have significant association with the trait. Results indicate that the allelic contribution to the QTL located on chromosome 2A was predominantly from the low-Na uptake parent. Robust molecular markers for this locus are being developed.

Concluding remarks

Transformation techniques available for most crop species make it possible to manipulate the expression of genes involved in the control of transport of Na^+ across membranes. There are various candidate genes from higher plants, as indicated in Figure 4, and also some yeast-specific ones (Schachtman and Liu, 1999), for control of transport of Na^+ across membranes.

Some of these have been overexpresssed in model systems, with subsequent increase in salt tolerance. The most dramatic responses have been with the vacuolar antiporter AtNHX1 in *Arabidopsis* (Apse et al., 1999) and tomato (Zhang and Blumwald, 2001).

However, the lack of community acceptance of genetic engineering of crop species means that other approaches need to be taken at present. Molecular markers offer a way around this current impasse; user-friendly markers can be developed from germplasm with contrasting phenotypes, i.e., quantifiable trait differences, using QTL or bulk segregant analysis, or from genes of known function. They can then be used to follow the inheritance of the trait during backcrossing into cultivars.

We conclude that there is considerable natural genetic variation in transport processes controlling the uptake and accumulation of Na^+ and Cl^- that is yet to be utilised for increasing the salt tolerance of crop species. With an understanding of the function of these transporters at the whole plant level, this genetic variation can be exploited for developing molecular markers to track the introduction of salt-tolerant germplasm into cultivars by conventional breeding methods, and ultimately for identifying genes that can be used for transformation when salt tolerance in closely related germplasm cannot be found.

In summary, modern molecular techniques offer new approaches to improving salt tolerance of crops. Possibly a combination of all approaches, old and new, will be the most productive. Identifying physiological traits and key genes, and understanding mechanisms at the cellular and whole plant level, is central to all approaches.

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