

FOCUS PAPER

## Improving crop salt tolerance

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### Abstract

Salinity is an ever-present threat to crop yields, especially in countries where irrigation is an essential aid to agriculture. Although the tolerance of saline conditions by plants is variable, crop species are generally intolerant of one-third of the concentration of salts found in seawater. Attempts to improve the salt tolerance of crops through conventional breeding programmes have met with very limited success, due to the complexity of the trait: salt tolerance is complex genetically and physiologically. Tolerance often shows the characteristics of a multigenic trait, with quantitative trait loci (QTLs) associated with tolerance identified in barley, citrus, rice, and tomato and with ion transport under saline conditions in barley, citrus and rice. Physiologically salt tolerance is also complex, with halophytes and less tolerant plants showing a wide range of adaptations. Attempts to enhance tolerance have involved conventional breeding programmes, the use of *in vitro* selection, pooling physiological traits, interspecific hybridization, using halophytes as alternative crops, the use of marker-aided selection, and the use of transgenic plants. It is surprising that, in spite of the complexity of salt tolerance, there are commonly claims in the literature that the transfer of a single or a few genes can increase the tolerance of plants to saline conditions. Evaluation of such claims reveals that, of the 68 papers produced between 1993 and early 2003, only 19 report quantitative estimates of plant growth. Of these, four papers contain quantitative data on the response of transformants and wild-type of six species without and with salinity applied in an appropriate manner. About half of all the papers report data

on experiments conducted under conditions where there is little or no transpiration: such experiments may provide insights into components of tolerance, but are not grounds for claims of enhanced tolerance at the whole plant level. Whether enhanced tolerance, where properly established, is due to the chance alteration of a factor that is limiting in a complex chain or an effect on signalling remains to be elucidated. After ten years of research using transgenic plants to alter salt tolerance, the value of this approach has yet to be established in the field.

Key words: Halophytes, salt tolerance, transgenic plants.

### Why should we want to improve crop salt tolerance?

Earth is a salty planet, with most of its water containing about 30 g of sodium chloride per litre. This salt solution has affected, and continues to affect, the land on which crops are, or might be, grown. Although the amount of salt-affected land (about  $900 \times 10^6$  ha) is imprecisely known, its extent is sufficient to pose a threat to agriculture (Flowers and Yeo, 1995; Munns, 2002) since most plants, and certainly most crop plants, will not grow in high concentrations of salt: only halophytes (by definition) grow in concentrations of sodium chloride higher than about 400 mM. Consequently, salinity is a threat to food supply. Although there is currently food enough for the world population, more than 800 million people are chronically undernourished (Conway, 1997). Growth of the human population by 50%, from 6.1 billion in mid-2001 to 9.3 billion by 2050 (<http://www.unfpa.org/swp/2001/>), means that crop production must increase if food security is to

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be ensured, especially for those who live on about \$1 per day.

Approximately half of the world's land surface is 'perennial desert or drylands' (United Nations Development Programme, see <http://www.undp.org/seed/unso/pub-htm/dryland-population.pdf>). These areas can only be made more productive by irrigation (and irrigation increased by almost one-third between 1979 and 1999, from 207 to 274 million hectares; <http://apps.fao.org/page/collections?subset=agriculture>). Unfortunately, a strong link with salinization (Ghassemi *et al.*, 1995), throws an immediate question over the sustainability of using irrigation to increase food production and it has been argued elsewhere (Shannon and Noble, 1990; Flowers and Yeo, 1995) that the primary value of increasing the salt tolerance of crops will be to the sustainability of irrigation. Given the amount by which food production will have to be increased, it seems reasonable to predict that changing the salt tolerance of crops will be an important aspect of plant breeding in the future, if global food production is to be maintained.

### Historical perspective

The need to produce salt-tolerant crops was evident in ancient times (Jacobsen and Adams, 1958) and the possible ways to increase tolerance have been extensively rehearsed. Epstein *et al.* (1980) described technical and biological 'fixes' to the problem of salinity. The 'biological fix' was founded on salt tolerance having a genetic basis, for which the evidence (Epstein *et al.*, 1980) was the existence of a salt-tolerant flora (halophytes) and differences in salt tolerance between genotypes within species. Varietal differences in salt tolerance have been known since the 1930s (Epstein, 1977) and intraspecific selection for salt tolerance was, by the 1980s, shown to be possible with rice (Akbar and Yabuno, 1977) and barley (Epstein *et al.*, 1980).

In spite of early promise, the 'biological fix' has been slow in arriving. In 1993, Flowers and Yeo (1995) reviewed the evidence for the paucity of new salt-tolerant cultivars and concluded that the number was likely to be fewer than 30. Since 1993, there have been just three registrations of salt-resistant cultivars in *Crop Science* (Owen *et al.*, 1994; Al-Doss and Smith, 1998; Dierig *et al.*, 2001) and one patent registered in the US (Dobrenz, 1999); one other patent claims a method to increase tolerance in cereals by the incorporation of a late embryogenesis abundant (LEA) protein (Wu and Ho, 1996). Flowers and Yeo (1995) concluded that, although salinity might be of profound local importance, it had not yet had sufficient impact on regional agricultural production to warrant the effort necessary to produce new salt-tolerant cultivars.

## The complexity of salt tolerance

### Assessment of tolerance

Ultimately, salt tolerance of crops is tested as yield from farmers' fields. However, evaluating field performance under saline conditions is notoriously difficult because of the variability of salinity within fields (Richards, 1983; Shannon and Noble, 1990; Daniells *et al.*, 2001) and the enormous potential for interactions with other environmental factors, ranging from gaseous pollutants, soil fertility and drainage to temperature, light flux density and transpirational water loss. Consequently, prediction of 'field' performance is commonly carried out in trial plots, or using a solution-based method where the salinity of the medium can be readily adjusted to required values (Maas and Hoffmann, 1977; Francois and Maas, 1994). The latter often precludes measuring yield through lack of space and estimates of tolerance obtained from such experiments may not always be borne out by the response of plants in the field (Rowland *et al.*, 1989; Daniells *et al.*, 2001). Evaluating tolerance is made more complex by variation in sensitivity to salt during the life cycle. For example, it has long been known that grain yield in rice is much more depressed by salt than is vegetative growth (Khatun and Flowers, 1995): germination is relatively salt resistant. In tomato, tolerance at germination is not correlated with the ability to grow under salt stress: both are controlled by different mechanisms (Foolad and Lin, 1997), although it is possible to find genotypes with similar tolerance at germination and during vegetative growth (Foolad and Chen, 1999).

### Genetics of salt tolerance

Perhaps the first attempt to evaluate the inheritance of salt tolerance was made by Lyon (1941). An interspecific cross of *Lycopersicon esculentum* and *L. pimpinellifolium* showed fruit yield of the hybrid was more sensitive to increasing salt (sodium sulphate) than that of either parent. Other crosses of wild and cultivated tomato also suggested a complex genetics. Heterosis was apparent under saline (NaCl) conditions in the elongation of stems in hybrids of *L. esculentum* produced with three wild species (*L. cheesmanii*, *L. peruvianum*, and *L. pennellii* = *Solanum pennellii*) by Tal and Shannon (1983). Stem elongation was a dominant trait in hybrids with *S. pennellii*, but not with *L. cheesmanii* as the parent. Total dry matter production of another F<sub>1</sub> hybrid, between *L. esculentum* and *L. pennellii*, showed hybrid vigour (Saranga *et al.*, 1991) under saline conditions. Analysis of other species has also suggested that the genetics of salt tolerance is complex.

In rice, sterility, an important factor in yield under saline conditions, is determined by at least three genes (Akbar *et al.*, 1972; Akbar and Yabuno, 1977). In diallel analysis the effects of salinity on the seedling stage and on sterility

suggested both additive and dominance effects, some with high heritability<sup>†</sup> (Moeljopawiro and Ikehashi, 1981; Akbar *et al.*, 1986). Evidence of dominance of tolerance is also seen with pigeonpea (*Cajanus cajan*), where a cross with *Atylosia albicans* (one of the most salt-tolerant relatives of pigeonpea) produced intergeneric hybrids that behaved as the wild parent, indicating dry weight production was determined by a dominant genetic factor (Subbarao *et al.*, 1990). There is also evidence of dominance in the salt tolerance of sorghum. Diallel analysis, based on assessing tolerance to NaCl as relative root length in salt-treated as compared with control plants, showed that there were both additive and dominance effects of NaCl (Azhar and McNeilly, 1988). These examples suggest that while the assessment of tolerance is complicated by changes occurring during the ontogeny of a plant and may be technically difficult under field conditions, there is evidence of a genetically complex trait (Shannon, 1985), showing heterosis, dominance and additive effects.

#### Physiological complexity

As well as the genetic evidence, there is physiological evidence to support the view that salt tolerance is a complex trait. Halophytes show a wide range of adaptations from the morphological to the biochemical (Flowers *et al.*, 1986; Leach *et al.*, 1990; Flowers and Dalmond, 1992; Glenn *et al.*, 1999; Tester and Davenport, 2003), adaptations that include the ability to remove salt through glandular activity. Although control of ion uptake is exercised at the root, the ability to secrete ions has evolved into a successful strategy for salt tolerance. Some (but by no means all) halophytes utilize salt-secreting glands to remove excess ions from their leaves (Thomson *et al.*, 1988), reducing the need for very tight balancing of ion accumulation and growth (Flowers and Yeo, 1988). Within less tolerant species, intraspecific variation in tolerance is also associated with variation in a wide variety of physiological traits (Yeo *et al.*, 1990; Cuartero *et al.*, 1992; Foolad, 1997; Wahid *et al.*, 1997; Tozlu *et al.*, 1999a, b).

Perhaps the best investigated of the traits relating to salt tolerance are those associated with the ion contents of plants grown in the presence of salts. Although there was an early hint that an ability to exclude chloride in *Glycine* showed simple Mendelian inheritance (Abel, 1969), there proved to be complications introduced by interactions with

phosphate (Grattan and Maas, 1984, 1988). In vine, chloride exclusion appeared to be inherited either as a qualitative or a quantitative trait, depending on the parents (Sykes, 1992), while in inter-specific crosses of *Citrus*, chloride accumulation showed continuous variation amongst progeny suggesting that this is a polygenic trait, although with a strong heritable basis (see Sykes, 1992). In *Trifolium repens*, heritability of chloride accumulation, estimated from parent–progeny regressions, is only moderate (0.24–0.37, Rogers *et al.*, 1997), but sufficient for net accumulation of chloride to be a useful tool in the selection of salt-tolerant genotypes.

For the other dominant ion in saline soils, sodium, there has been considerably more research, often associated with an estimation of the other major monovalent cation in plants, potassium. The tolerance of plants to sodium chloride is commonly, but not uniquely, related to the concentration of sodium in the shoot. For tomato, Foolad (1997) reported, from a parent–progeny comparison, that sodium accumulation under saline conditions was under genetic control, with more than 90% of the genetic variation attributable to additive effects: dominance had little influence. Analysis of sodium and potassium accumulation between  $F_n$  and  $F_{n+1}$  families of rice growing under saline conditions showed that net accumulation of both sodium and potassium to be heritable (with narrow sense heritabilities of between 0.4 and 0.5), although shoot sodium and potassium concentrations were unrelated, suggesting that the pathways for net accumulation of sodium and potassium in rice are separate (Garcia *et al.*, 1997a). A high degree of heterosis and large environmental effects on Na/K ratios (Gregorio and Senadhira, 1993) are characteristic of this aspect of salt tolerance in rice behaving as a quantitative trait.

The means by which sodium enters plants is still poorly understood. At low external concentrations, potassium may enter roots through K carriers, while at higher concentrations, non-selective cation channels (Demidchik *et al.*, 2002) are possible means of transport. Channels that are activated when the transmembrane potential is hyperpolarized are highly selective for K; other channels, activated when the membrane potential is depolarized, are less selective and could be one means by which sodium enters cells (Maser *et al.*, 2002b). Sodium can also enter via KUP/HAK/KT potassium transporters, cyclic-nucleotide-gated channels, glutamate-activated channels, LCT transporters, and HKT transporters, although the relative roles of each seem likely to vary across species (Maser *et al.*, 2002b; Tester and Davenport, 2003). HKT1 plays a role in net Na accumulation into wheat (Laurie *et al.*, 2002) and into the distribution of ions between roots and shoots of arabidopsis (Maser *et al.*, 2002a). The rice *OsHKT1* is down-regulated after osmotic shock (with 150 mM NaCl) of plants growing in a low (micromolar) potassium

<sup>†</sup> Most estimates of the heritability of complex traits are made from variance ratios. Where genetic and phenotypic variability are estimated simply from comparisons of varietal performance, then broad sense heritability is obtained from the ratio of the variance within genotypes to the sum of the genetic, environmental and genetic×environmental variances. In experiments where a trait is compared in parents and progeny, it is possible to split the genetic variance into additive and dominance effects: this allows the calculation of narrow sense heritability (Simmonds, 1979).

concentration and more so in a vigorous tolerant landrace than in a sensitive dwarfed variety (Golldack *et al.*, 2002).

Much has been written about the importance of the ability of plants to discriminate between sodium and potassium, for which a simple index, the K/Na ratio, can be determined for plants and plant parts. In bread wheat, the discrimination between potassium and sodium in their transport to the shoot, manifested as K/Na ratio in shoot tissue, is apparently determined by a locus described as *Kn1* and confirmed by RFLP analysis to be completely linked to five markers on the long arm of Chromosome 4D (Gorham *et al.*, 1997). That the ratio of K to Na in a plant is determined at a single locus, if proved true, is surprising, given the number of proteins that might contribute to Na and K transport from root to shoot, unless they, or their control, are clustered in a particular chromosomal location. The K/Na discrimination trait can be transferred from durum to bread wheat (Dvorak *et al.*, 1994). However, control of the K/Na discrimination itself cannot be confined to the D genome, as in durum wheat (*Triticum turgidum* L. ssp *durum*) discrimination equivalent to that found in the hexaploid bread wheat has been found in lines which contain no D genome (Munns *et al.*, 1999).

#### *Quantitative trait loci*

There is, then, considerable evidence to support the view that salt tolerance and its sub-traits might be determined by multiple gene loci. In an intergeneric cross of tomato, quantitative trait loci (QTL) were found associated with fruit yield in plants growing under saline conditions (Breto *et al.*, 1994), although some of the QTL identified were later shown to be dependent on the parentage of the cross (Monforte *et al.*, 1997a). An important conclusion stemming from this work was that QTL are treatment-sensitive. Some QTL associated with aspects of fruit yield were found regardless of whether the plants were grown with or without salt; others were detected only under saline or under non-saline conditions (Monforte *et al.*, 1997b). Other crosses have also identified both stress- (salt and cold) specific and stress-non-specific QTL: the stress-non-specific QTL generally exhibited larger individual effects and accounted for a greater portion of the total phenotypic variation under each condition than the stress-specific QTL (Foolad *et al.*, 1999). As for the QTL identified for fruit yield, QTL associated with germination depend upon the conditions under which germination is assessed (Foolad *et al.*, 1999). A similar situation exists for citrus, where about half of the potential QTL identified depended on the presence or absence of salinity (Tozlu *et al.*, 1999a), and in rice (Gong *et al.*, 1999, 2001) where less than 10% of the QTL were detected both in the presence and absence of salt. Clearly, the major determinants of yield vary with the environmental conditions and quantitative traits typically exhibit a large environment  $\times$  genotype interaction.

The use of tomato has also been important in establishing that QTL associated with tolerance vary with the stage of plant development. The QTL associated with tolerance at germination (Foolad *et al.*, 1997, 1998) and vegetative growth (Foolad and Chen, 1999; Foolad *et al.*, 2001) differ (Foolad, 1999). Such differences are not restricted to tomato and have been demonstrated in arabidopsis (Quesada *et al.*, 2002) and cereals; both barley (Mano and Takeda, 1997) and rice (Prasad *et al.*, 2000). QTL associated with aspects of ion transport have also been reported in citrus (Tozlu *et al.*, 1999b) and in rice (Koyama *et al.*, 2001).

#### *Conclusions*

There is sufficient evidence to be confident that salt tolerance is a multigenic trait. Research on the physiology of salt tolerance suggests that the overall trait is determined by a number of sub-traits any of which might, in turn, be determined by any number of genes. These sub-traits generally include an ability to minimize the net accumulation of sodium and/or chloride ions and to select potassium from a background of high sodium concentration.

#### **Approaches to enhancing tolerance**

Flowers and Yeo (1995) suggested five possible ways, which were appropriate at that time, to develop salt-tolerant crops: (1) develop halophytes as alternative crops; (2) use interspecific hybridization to raise the tolerance of current crops; (3) use the variation already present in existing crops; (4) generate variation within existing crops by using recurrent selection, mutagenesis or tissue culture, and (5) breed for yield rather than tolerance. These all remain possible solutions to the problem. Although conventional forms of mutagenesis have not, in general, delivered salt-tolerant genotypes (Flowers and Yeo, 1995; but see Tester and Davenport, 2003), mutagenesis has unearthed a number of salt-sensitive types (Borsani *et al.*, 2002; Zhu, 2002). Bohnert and Jensen (1996) claimed that an important approach had been missed by Flowers and Yeo: they wrote 'tolerance breeding must be accompanied by transformation'; and that 'successful releases of tolerant crops will require large-scale "metabolic engineering" which must include the transfer of many genes'. While such an approach was not feasible in the early 1990s (Flowers and Yeo, 1996); this approach is now being widely advocated. Some 13 species (Table 1A) have been transformed with nearly 40 genes in experiments reported between 1993 and 2003 (Table 1B). The majority of experiments have used rice, tobacco and arabidopsis; transformations involving the synthesis of compatible solutes have been more popular than any other, with those involving glycine betaine the most commonly performed (Table 1A). There is an increasing number of

**Table 1.** Species and genes used in the transformation of plants where authors claimed enhancement of salt tolerance

(A) Species	No. of experiments reported
<i>Arabidopsis thaliana</i>	14
<i>Brassica napus</i> and <i>B. juncea</i>	3
<i>Citrus</i> (Carrizo citrange)	1
<i>Cucumis melo</i> (melon)	2
<i>Diospyros kaki</i> (Japanese persimmon)	1
<i>Lycopersicon esculentum</i> (tomato)	5
<i>Medicago sativa</i> (alfalfa)	2
<i>Nicotiana tabaccum</i> (tobacco)	19
<i>Oryza sativa</i> (rice)	17
<i>Solanum melongena</i> (eggplant)	1
<i>Solanum tuberosum</i> (potato)	2
<i>Triticum aestivum</i> (wheat)	
(B) Transformed for	No. of experiments
Apoplastic invertase, Apo-Inv	1
Arginine decarboxylase, ADC	1
Betaine aldehyde dehydrogenase, BADH; betB, choline dehydrogenase (CDH); choline oxidase, codA (glycinebetaine)	15
Ca <sup>2+</sup> -dependent protein kinase, CDPK	1
Ca/H antiporter, CAX1	1
Calcium-binding protein, EhCaBP	1
Calicneurin; protein kinase, CaN	1
Ca protein kinase, OsCDPK7	1
Glutathione S-transferase, GST and glutathione peroxidase, GPX	1
Glyceraldehyde-3-phosphate dehydrogenase, GPD	1
Glycogen-synthase kinase-3, AtGSK1	1
Glutamine synthetase, GS2	1
Heat shock protein, DnaK/HSP70	1
High-affinity potassium transporter, *HKT1 <sup>a</sup>	3
Isopentenyl transferase, ipt (increased cytokinin)	1
Late embryo abundant protein, HVA1 (a LEA)	2
Mannitol 1-phosphate dehydrogenase, mt1D (mannitol)	6
Myo-inositol O-methyltransferase, IMT1 (ononitol)	1
Omega-3 fatty acid desaturase, <i>fad7</i> (fatty acid processing)	1
Osmotin-like protein	1
Proline dehydrogenase; Delta (1)-pyrroline-5-carboxylate synthetase (proline)	4
Proline transporter, <i>AhProT1</i>	1
Proton sodium exchanger, *HNX1 <sup>a</sup>	4
Putative transcription factor, Alfin1	2
Rare Cold Inducible gene 3, RCI3	1
Rice <i>Hal2</i> like, <i>RHL</i>	1
S-adenosylmethionine decarboxylase, SAMDC (spermine, spermidine)	1
Serine/threonine kinase, AT-DBF2	1
Sorbitol-6-phosphate dehydrogenase, SPD (sorbitol)	1
SR-like, putative splicing protein	1
Transcription factors, DREB1A; AhDREB1	2
Trehalose-6-phosphate synthase/phosphatase, TPSP (trehalose)	1
Yeast halotolerance gene, <i>Hal2</i>	3
Yeast halotolerance gene, <i>Hal1</i>	2
Yeast mitochondrial superoxide dismutase, Mn-SOD	1
Vacuolar H <sup>+</sup> -pyrophosphatase, AVP1	1

<sup>a</sup> Asterisk indicates a specific prefix, e.g. *At*.

claims in this literature that overall tolerance can be manipulated through alteration in the activity of one or two genes (see below), which was not something claimed by Bohnert and Jensen (1996). For a trait as complex as salt tolerance this seems intuitively unlikely. The fundamental issue to be resolved is the importance of individual components or sub-traits of salt tolerance and whether the manipulation of individual or of many genes is required to alter complex traits. If altering a single gene can alter

tolerance, this suggests either that changing the concentration of a few key components has a substantial effect on a wide range of other processes or that salt tolerance is not as complex as it appears or that a key limit to tolerance might be altered in any given species (or genotype). Substantiating, or otherwise, claims that tolerance is altered by transformation is clearly of major importance both for our understanding of complex traits and for the practicalities of their manipulation.

**Table 2.** Possible combinations for experiments reporting the evaluation of transgenic plants

Column 1 lists experimental sites and column 2 the parameters that might be evaluated—yielding six possible combinations. In each combination, either quantitative or qualitative data might be reported on transformant and/or wild type, ideally under saline and non-saline conditions, but often only the transformant under saline conditions. Ideally, an experiment evaluating the effects of a transformation would report quantitative data on yield of field grown plants of both wild-type and transformant lines in the presence or absence of salinity.

Experimental location	Parameter evaluated	Data	Material tested	Treatments
1	2	3	4	5
Field	Yield	Quantitative	Transformant lines	Plus salinity
Greenhouse or other controlled environment	Growth	Qualitative	Wild type	Minus salinity
'In vitro' (including germination)				Plus and minus salinity

### Genetic engineering of salt tolerance: evaluation of success

The evaluation of transgenic material requires some comment. The material to be tested should be genetically stable (it has been suggested that it should be in its fourth or fifth generation by Bajaj *et al.*, 1999) and a comparison of as many transformed lines as possible made with the performance of the parental (wild-type) line under saline and non-saline conditions (Table 2). It is important to know whether or not the overall growth of the transgenic plant has been affected, as vigour itself is an important determinant of salt tolerance. For crops, claims of enhanced tolerance should be made on the basis of yield. Unfortunately, there were no such reports by 1999 (Bajaj *et al.*, 1999) and the situation had changed little by early 2003. Given the paucity of data on crop yield (just five reports of estimates of crop yields, Guo *et al.*, 1997; Wang *et al.*, 2000; Zhang and Blumwald, 2001; Zhang *et al.*, 2001; Li *et al.*, 2002), the success, or otherwise, of a transformation in altering salt tolerance has generally to be evaluated against the nature of the data that is presented. Those claims based on quantitative estimates of the growth of fourth or fifth generation transgenic lines should be seen as stronger than claims based upon photographic evidence of the performance of plants of the primary transformants grown in salt alone. In the following analysis, papers are allocated to one of five categories (Table 3). Only those data relating to the growth of plants under conditions in which transpiration occurs have been evaluated: it is transpiration that transports ions to the shoots, where their presence brings about injury and death. Photographs of plants in culture medium are unconvincing as evidence for a successful alteration of crop yield. Quantitative measures of growth are required for plants grown in the presence and absence of salt: the ability to germinate in salt is, in general, a poor indicator of performance in the field. It is also important that salt be added in such a way that it is not the effect of water or osmotic stress that is being evaluated and this generally requires an increase of salt concentration of 50 mM or less per day and determination of the

consequences days or weeks later, depending on the salt tolerance of the species (Munns, 1993, 2002).

Analysis of publications to date shows that of the 68 reports produced between 1993 and early 2003 (Table 3; see also supplementary data online) only 19 describe quantitative estimates of plant growth. Of these, four papers (Table 4) contain quantitative data on the response of transformants and wild type of six species without and with salinity applied in an appropriate manner. About half of all the papers (35, Table 3 and supplementary data online) report data on experiments conducted under conditions where there is little or no transpiration: such experiments may provide insights into components of tolerance, but are not grounds for claims of enhanced tolerance at the whole plant level—in such a system, the fern *Ceratopteris*, where single gene mutants alter the salt tolerance in the gametophytic generation (Warne *et al.*, 1995) might be a useful genetic model.

Those experiments where the effects of transformation were determined in saline and non-saline soil or hydroponic culture suggest that real changes in salt tolerance can be effected, but generally not without consequences for the growth that occurs in the absence of salt. Over-expression of the gene *Alfin1* in alfalfa increased its salt tolerance and promoted root growth and shoot growth (Table 4A), under normal and saline conditions, producing larger plants than the wild type (Winicov, 2000). *Alfin1* is a putative transcription factor, but its mode of action in altering overall salt tolerance is still unclear. Adding to the uncertainty of how some genes affect overall tolerance is the consequence of transforming tomato with the yeast gene *HAL1*. *HAL1* alters the salt tolerance of tomato (Gisbert *et al.*, 2000) and increases the K/Na ratio in transgenic plants. However, these transgenic plants, when grown in the absence of salt, had half the shoot dry weight of the wild type (Table 4A). A similar effect of an introduced gene on growth is also seen following the transformation of tobacco with mannitol-1-phosphate dehydrogenase. Here mannitol that accumulated as a consequence of the transformation made only a small contribution to the osmotic potential of the transformed

**Table 3.** Criteria used to define categories of experiments where an enhancement of salt tolerance was claimed as a consequence of a transformation for plants and the numbers of experiments reported (between 1993 and early 2003) in each of those categories

Criteria			Category	Number in category
Data	Material tested	Treatments		
Quantitative	Transformants and wild type	Plus and minus salinity	1	6
Quantitative	Material missing	Treatments missing	1a	13
Qualitative	Transformants and wild type	Plus and minus salinity	2	2
Qualitative	Material missing	Treatments missing	2a	12
All <i>in vitro</i>			3	35

**Table 4.** The effects of transformation (A) and hybridization (B) on the growth (g dry weight or fresh weight) or yield of plants in the presence and absence of salt

Species	Minus NaCl		Plus NaCl			Reference
	Wild type	Transformant	Wild type	Transformant	NaCl (mM)	
(A)						
Alfalfa	0.50	2.18	–	–	–	Winicov, 2000 <sup>a</sup>
Tomato	1.4	0.67	0.70	0.72	150	Gisbert <i>et al.</i> , 2000 <sup>b</sup>
Tobacco	73.6	55.0	40.4	41.5	150	Karakas <i>et al.</i> , 1997 <sup>c</sup>
<i>Arabidopsis</i>	0.311	0.189	0.05	0.052	100	Huang <i>et al.</i> , 2000 <sup>d</sup>
Canola	1.51	0.60	1.09	0.60	300	Huang <i>et al.</i> , 2000 <sup>d</sup>
Tobacco	1.174	0.231	0.661	0.209	150	Huang <i>et al.</i> , 2000 <sup>e</sup>
	Cultivated parent	Hybrid	Cultivated parent	Hybrid		
(B)						
Wheat	121.2	75.4	23.0	21.2	150	King <i>et al.</i> , 1997 <sup>f</sup>
Tomato	7438	5132	2343	1244	150	Rush and Epstein, 1981 <sup>g</sup>

<sup>a</sup> Weighted mean of three transformed lines.

<sup>b</sup> Shoot dry weight.

<sup>c</sup> Total PLANT dry weight.

<sup>d</sup> Dry weight per plant: choline-supplemented wild-type (WT) and betaine-producing transgenic lines.

<sup>e</sup> Fresh weight per plant: choline-supplemented wild-type (WT) and betaine-producing transgenic lines.

<sup>f</sup> Grains per plant.

<sup>g</sup> Average fruit yield, g fresh weight per plant.

plants, which were smaller than the wild-type, although they were less affected in relative terms by salinity (Table 4A). A similar situation was reported by Huang *et al.* (2000) for *Arabidopsis*, canola and tobacco transformed to oxidize choline to glycinebetaine (Table 4A). In all of these cases, the effects of the genes are not simply on tolerance, making the evaluation of the effects complex. Tolerance, judged in relative terms (i.e. yield in the saline conditions expressed as a proportion of yield in non-saline conditions), although an important indicator, is unlikely to impress a farmer unless the absolute yield is adequate. A genotype whose yield is hardly affected by salinity may well still be out-performed by a vigorous, high-yielding genotype which loses 50% of its yield under saline conditions, if the 'salt-tolerant' genotype is intrinsically low-yielding (Dewey, 1962). A similar situation has been previously reported for some hybrids between established crop varieties and wild relatives (Table 4B).

There is other, albeit weaker, evidence that transformation of plants with genes whose products affect transcription does appear to alter salt tolerance. Tobacco transformed with a gene isolated from a cDNA library prepared from salt-treated plants of *Atriplex hortensis*, by screening with a fragment of *Arabidopsis AtDREB2A* encoding a DNA-binding domain, apparently increased the tolerance of tobacco to salt (Shen *et al.*, 2003) without causing a dwarf phenotype, although quantitative data are lacking. A yeast kinase (a functional homologue of the yeast Dbf2 kinase) enhanced tolerance of tobacco cells to salt in tissue culture (Lee *et al.*, 1999). However, the assessment of salt tolerance in tissue culture is a poor predictor of tolerance in the whole plant and tolerance in cultured cells is not translated to tolerance in plants in the field (Flowers and Yeo, 1995). Transformation of *Arabidopsis* with the protein kinase coded by *AtGSK1* (a GSK/Shaggy-like protein kinase) induced anthocyanin

synthesis, a symptom of NaCl stress, in the absence of NaCl (Piao *et al.*, 2001) and promoted survival in soil irrigated with NaCl (but with 300 mM, which is likely to have produced an initial osmotic shock rather than a salt stress).

Recent research has shown that rice, transformed to overexpress genes that brought about the synthesis of trehalose, contained a reduced concentration of Na in the shoot and grew better than non-transformed (control) plants when in the presence of 100 mM NaCl (Garg *et al.*, 2002). Trehalose concentrations in the plants grown in 100 mM NaCl were relatively low, at less than 0.1 mg g<sup>-1</sup> fresh weight (approximately 5 mM in the cytoplasm if this were 10% of the water volume; Flowers *et al.*, 1991). It has been suggested that solutes such as trehalose are likely to function through their ability to scavenge reactive oxygen species (Zhu, 2001) and the protection afforded to the machinery of protein synthesis may be particularly important for normal repair processes (Chen and Murata, 2002). Another possible explanation for at least some of the compounds such as trehalose is that they act in a signalling cascade. Although trehalose is commonly present in bacteria, fungi and insects, its concentration in plants is very low and it may even be toxic: recent evidence suggests that this toxicity may stem from its role in the regulation of carbon metabolism (Muller *et al.*, 1999; Wingler, 2002). Earlier research on tobacco had shown that transformants producing trehalose were stunted in growth (Romero *et al.*, 1997) and experiments on rice had shown that treatment of plants with exogenous trehalose reduced sodium accumulation, but had a significant effect on root morphology (Garcia *et al.*, 1997b). In the more recent experiment of Garg *et al.* (2002), the synthesis of trehalose was under the influence of a stress-inducible promoter, so that growth under control conditions was presumably no different from the wild type (the authors note that non-stressed plants appeared normal, but did not, unfortunately, support this with quantitative data). The use of stress-inducible promoters may be an important way in which to avoid inhibition under non-stressed conditions (Kasuga *et al.*, 1999), if there are yield penalties from expressing genes under a constitutive promoter.

Although the targets of genetic engineering have largely been compatible solutes, there have been some attempts to manipulate one of the underlying causes of salt damage, the net accumulation of sodium ions. Down-regulation of HKT1 in wheat increased resistance to salinity under conditions of low K supply (Laurie *et al.*, 2002) and transformation of the cyanobacterium *Synechococcus* with a Na/H antiporter increased its tolerance to salt (Waditee *et al.*, 2002). For higher plants, however, any enhancement of antiporter activity would have to be targeted to root cells, for in the aerial parts of the plant enhanced Na/H antiporter activity would only exacerbate the consequences of ion accumulation in cell walls (Oertli, 1968; Flowers

*et al.*, 1991; Munns, 2002). Even in the roots, it is likely that ions removed from cortical cell walls would have osmotic consequences (Yeo, 1998): only in situations where there was a large volume of external solution (e.g. marine algae) would there be a chance of ions effluxing from the cytoplasm being washed from cell walls. Manipulating the vacuolar proton gradient to enhance ion accumulation has also led to claims of enhanced salt tolerance in transgenic plants (Gaxiola *et al.*, 2002). However, there is only qualitative evidence for *Arabidopsis* (Apse *et al.*, 1999) and the evidence obtained with *Brassica napus* (Zhang *et al.*, 2001) and tomato (Zhang and Blumwald, 2001) does not include (other than a photograph) the effects of salt (200 mM) on the wild type. In other experiments, *B. napus* continued to yield in 200 mM NaCl (Ashraf *et al.*, 2001) as does the tomato cultivar MoneyMaker (TJ Flowers and SA Flowers, unpublished data). While the ability to accumulate sodium in leaf vacuoles is clearly a trait that is important for dicotyledonous halophytes, in such species this ability is coupled with other traits such as the regulation of transpiration, the synthesis of compatible solutes and an ability to function with low cytoplasmic potassium concentrations (Flowers and Dalmond, 1992).

#### *Conventional breeding programmes*

Strategies for breeding for salt tolerance in cross-pollinating species by cycles of recurrent selection were described long ago (Dewey, 1962): for a self-pollinating species the same process would require the use of male-sterile lines to facilitate out-crossing (Ramage, 1980). These approaches depend on adequate heritability of the overall trait, for which there is evidence for wild grasses (Ashraf *et al.*, 1986), sorghum (0.74, Maiti *et al.*, 1994), maize (0.4, Maiti *et al.*, 1996), and tomato (Saranga *et al.*, 1992, 1993). In both rice (Yeo *et al.*, 1988) and *Trifolium* (Rogers and Noble, 1992; Rogers *et al.*, 1997), it has proved possible to select lines whose ion contents, when grown under saline conditions, are either higher or lower than those of the parental types. By way of contrast, Saranga *et al.* (1992) concluded that for tomato (a cross between *L. esculentum* and *L. pennellii*), selection for ion contents would not improve the breeding process.

#### *Use of in vitro selection*

The use of *in vitro* selection was widely advocated during the 1980s, but did not result in cultivars in farmers' fields (Rowland *et al.*, 1989). More recently, selections for alfalfa (Winicov, 1991; Winicov and Bastola, 1997) look promising and there may be a use for somaclonal variants within breeding programmes (Zhu *et al.*, 2000).

#### *Pooling physiological traits*

The possibility of pooling physiological traits has been advocated for rice (Yeo *et al.*, 1990), screening methods

evaluated (Garcia *et al.*, 1995) and the approach proved successful in generating salt-resistant lines (Gregorio *et al.*, 2002). The methodology does not require a deep knowledge of the genetics of traits, merely that they display sufficient heritability and that suitable screening procedures can be developed. The methods may be applicable to crops other than rice (Cuartero *et al.*, 1992; Ellis *et al.*, 1997; Foolad, 1997; Isla *et al.*, 1998; Munns *et al.*, 2002).

#### *Interspecific hybridization*

The introduction of genes from wild salt-tolerant species has been explored for tomato (Rush and Epstein, 1981; Tal and Shannon, 1983; Saranga *et al.*, 1991; Perez Alfocca *et al.*, 1994), tomato/potato (Sherraf *et al.*, 1994), wheat (Dvorak and Ross, 1986; Gorham *et al.*, 1986; Mahmood and Quarrie, 1993; Martin *et al.*, 1993; William and Mujeebkazi, 1993; King *et al.*, 1997*a, b*), and pigeonpea (Subbarao *et al.*, 1990). However, the approach has not led to the release of salt-tolerant crops, although there is a recent proposal for a new salt-tolerant cereal, tritipyrum (King *et al.*, 1997*b*),

#### *Halophytes as alternative crops*

Historical evidence suggests that farmers shift from more sensitive to more tolerant crops as salinity in their fields rises (Jacobsen and Adams, 1958). The natural end of such a succession would be the use of halophytes, whose potential as crops has been explored (Malcolm, 1969; O'Leary, 1984; O'Leary *et al.*, 1985; Lovett, 1993; Troyodieguez *et al.*, 1994; Zahran, 1994; Brown and Glenn, 1999; Brown *et al.*, 1999; Glenn *et al.*, 1999), but is yet to be fully realized. Since the domestication of wild species was, in the past, a successful strategy, this must remain a useful approach for generating salt-tolerant crops in the future, especially given the wide range of halophytes available.

#### *Use of marker-aided selection*

The multigenic nature of salt tolerance has clearly been established and quantitative trait loci associated with aspects of germination, ion transport and yield. One obvious use of QTL in plant breeding for salt tolerance is in marker-aided selection (or marker-assisted selection, MAS). The drawbacks in using marker-assisted breeding are 'linkage drag' of undesirable traits due to the large size of regions of chromosomes identified by QTL (Asins, 2002) and the fact that environment and genetic background have a significant influence on the QTL that are identified (see above). In a wider context, QTL might be used to identify genes that are important in salt tolerance and it is noteworthy, given the complexity of salt tolerance, that so few QTL are identified (Yeo *et al.*, 2000) within any given genome. This may be an indication that traits are determined by a limited number of sites and/or that genes associated with physiological traits are clustered on

chromosomes. However, the fact that a QTL represents many, perhaps hundreds, of genes remains a problem to finding key loci within a QTL. The easiest way forward may be through the identification of candidate genes. Of the five QTL associated with the effects of salinity on vegetative growth in arabidopsis (Quesada *et al.*, 2002) one was located close to the location of *SOS2* (which codes for a serine/threonine protein kinase) and another close to the positions of *RD29A* and *RD29B* (genes coding for hydrophilic proteins involved in ABA signal transduction).

## Conclusions

It is conceivable that approaches that identify specific genes that are up- or down-regulated either through the analysis of RNA (Kawasaki *et al.*, 2001) or proteins (Salekdeh *et al.*, 2002) might provide a specific focus for transformation, although choosing key genes for tolerance is currently far from happening. Transgenic technology will undoubtedly continue to aid the search for the cellular mechanisms that underlie tolerance, but the complexity of the trait is likely to mean that the road to engineering such tolerance into sensitive species will be long. In the meantime, it would be expedient to continue to invest in other avenues such as the manipulation of ion excretion from leaves through salt glands and the domestication of halophytes. Experience suggests authors should avoid hyperbole in their titles and summaries, as this does little service to the long-term aim of improving the salt tolerance of crops in the field.

## Supplementary data

Table S1 is a list, in date order, of papers where plants have been transformed to investigate or alter their salt tolerance, together with the category (see Table 3) assigned to the paper. It can be found at *Journal of Experimental Botany* online.

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