Molecular biology of salt tolerance in the context of whole-plant physiology

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Abstract

The halobacteria are the only organisms that are tolerant of salinity at the molecular level. All other bacteria, all fungi, all plants, and all animals avoid the need for salt tolerance for most of their macromolecules by maintaining defined and conserved conditions in the cytoplasm. These conditions favour potassium over sodium, the limitation of total inorganic ion activity, and the supplementation of this where necessary with organic solutes which are metabolically neutral osmolytes that may also be osmoprotectant. The salt tolerance of an organism depends upon the range of external salinity over which it is able to sustain these conditions in the cytoplasm. There is substantial and increasing knowledge of the molecular biology and molecular genetics of the processes of ion and organic solute transport, solute synthesis, and compartmentation that underpin cell-based tolerance. Much of recent research focuses on the identification of genes and gene products that affect cell-based tolerance, commonly derived from single-cell models. There is commonly the implicit or explicit assumption that incorporation of these genes will benefit the salt tolerance of food crop species. While this essential experimental approach is giving enormous insight there should not be rash or premature expectations. The unique and overriding consideration for the salinity tolerance of terrestrial plants is the net flux of water due to transpiration and so resides at a higher level of organization. Processes that are advantageous to a single cell in an aqueous medium may be lethal to a cell in a leaf in the air. The likely impact of single structural-gene changes in ion and solute transport upon co-ordinated plant response is probably overestimated, and recent views consider regulatory processes and multiple gene transfers. While the technical ability for plant transformation increases daily, the practicality of using transgenic plants in complex breeding programmes seems rarely to be given enough thought. If intervention at the molecular level is to lead to salt-tolerant crop plants then it will be essential to view this in the contexts of whole plants and of plant breeding. Recent indications that a relatively small number of quantitative trait loci (QTL) may govern complex physiological characters offer the most hope for the future.

Key words: Salt tolerance, molecular biology, breeding programmes, QTL, whole-plant physiology.

Introduction

It has been recognized since the early nineteen-seventies (Flowers, 1972a, b; Greenway and Osmond, 1972) that higher plants do not have a salt-tolerant metabolism even if the organism itself thrives in seawater. The tolerance in vitro of general metabolic enzymes, of which malate dehydrogenase has been used as a typical example, does not differ between salt-sensitive crop species and those native to salt marshes. In complete contrast, the gene encoding malate dehydrogenase of the extremely halophilic archaebacterium Haloarcula marismortui, when cloned in Escherichia coli, produced a soluble but inactive protein, indistinguishable from the native protein from H. marismortui, but activated only by increasing the salt concentration to 3 M NaCl (Cendrin et al., 1993). All eukaryotic organisms differ diametrically from the halo-tolerant bacteria which have proteins whose primary amino acid sequence yields functional higher order structures only in the presence of high salinity in the protoplasm. Living cells of fungi, plants and animals universally maintain low cytoplasmic sodium ion concentrations even...
though the difference in free energy for sodium ions across the plasma membrane may be very great. Animal cells utilize active sodium ion extrusion to generate the free energy difference that is coupled to drive secondary symport and antiport carriers. Fungi and plants utilize primary active extrusion of protons rather than sodium ions, but their tolerance of cytoplasmic sodium is no greater. The conserved activation requirements of protein synthesis (Leigh and Wyn Jones, 1984) provide one account both for the need for ionic homeostasis favouring potassium over sodium in the cytoplasm of higher plants as well as obviating any general selective advantage of having enzymes which are salt-tolerant at the molecular level. There are exceptions in respect of certain classes of enzymes: such as those localized in the apoplast (Thiyagarajah et al., 1996) or catabolic enzymes that may be active in dying cells. These classes of enzyme are salt-tolerant irrespective of the salt tolerance of the species from which they derive. Such tolerance need have nothing to do with salinity, rather a generalized adaptation to operating in environments much less regulated than the living cytoplasm. There may be other specific exceptions in respect of membrane-spanning proteins which have a domain outside the regulated cytoplasm, be it in a plant cell wall, a vacuole, the sea or a gut. Other than these exceptions, salt tolerance in all organisms other than the halobacteria depends not upon salt-tolerant proteins per se, but upon conserving a defined micro-environment in the cytoplasm, regulated in respect to both the quantity and quality of inorganic ions.

There are many similarities at the cellular level between different organisms be they plant, fungi, animal, single-celled or multi-cellular. This has been the background to a reductionist approach favouring the use of yeast and bacterial models (Serrano and Gaxiola, 1994) and cell culture models (Hasegawa et al., 1994). There are also features unique to plants that differentially affect their reaction to a saline growth environment. These certainly interact with, and may outweigh, the role of certain cell-based processes.

Life is based upon water and salinity is a high concentration of certain dissolved ionic species. The quantity of water an organism uses dictates the powers of separation required between solute (water) and solvent (in this case inorganic ions). For aqueous environments (aquatic organisms, or any cell suspension) or in vitro culture with very limited transpiration there is little if any net flux of water. On dry land it is different. The exchange of oxygen and carbon dioxide is directly linked to the loss of water from the body of a plant or air-breathing animal because the concentration of water vapour in the leaf or the lung (at or close to saturation at leaf or body temperature) is greater than the concentration of water vapour in the atmosphere. Water vapour passes out along the same path as oxygen (animals) or carbon dioxide (plants) passes in. Animals take in oxygen and excrete carbon dioxide; their gas exchange is determined by the fact that the gas they need is present at about one part in five. The gas plants need is present at about one part in three thousand and this now puts plants at an immediate and a major disadvantage. To put this in perspective, an average human exercising vigorously would lose only percentage-points of bodyweight in water in an hour. A rapidly transpiring rice plant can change its leaf water ten times an hour in daylight (Yeo et al., 1997): 1000% of its bodyweight. Plants have to replace this water from a soil solution over which they have very little control (proton fluxes and phytosiderophores are some exceptions) and which will be 10% seawater in a mildly saline irrigation system and 100% seawater (and more) for the halophyte in a salt marsh.

Single-celled aquatic/marine organisms or cell cultures have a potentially limitless ‘outside’ into which anything can be excreted with relative impunity. The cells of complex animals can excrete into a bloodstream and have organs designed to deal with it. A plant root (except in hydroponics) can excrete only into the solution being drawn towards it by transpiration. A plant leaf cell has nowhere to excrete apart from the trivial solute-available volume of the apoplast except only in those small number of plants possessing specialized excretory glands.

A large, sometimes 90% or more, proportion of the volume of a plant cell is the internal vacuole. As well as providing size and shape and being a store for resources, the vacuole can also store toxic material or waste products. The vacuole takes over some of the functions that are unavailable in an organism that cannot excrete to the outside directly or via a bloodstream. In this sense a plant cytoplasm has two ‘outsides’ and its vital conditions have to be sustained in relation to the vacuole as well as to the exterior. Although subcellular compartmentation is universal and slowly-exchanging ionic compartments are found in single-celled yeasts (Nass et al., 1997) upwards, the large central vacuole is a feature of higher plant cells that has unique importance in salinity.

As well as cell-based similarities that extend across the kingdoms of living organisms, terrestrial plants face problems unparalleled in other organisms which drastically affect their relationship to saline environments. Much of our knowledge at the molecular level relates to the better-understood bacterial, fungal and animal systems, but its interpretation has to be made in the context of a plant with its roots in salty water, losing enormous volumes of water to the dry air; which is unavoidable in the attempt to acquire enough carbon dioxide to grow. As well as the difficulties faced by cells there are difficulties at higher levels of organization. Salt-sensitive plants suffer not because of the external salinity per se, but because growth falls below net ion import leading through ever-increasing internal concentrations to catastrophic failure (Munns
and Termaat, 1986; Yeo et al., 1991). In total contrast, halophytes thrive because growth and net salt uptake are tightly coupled, even if we do not know what controls what (Yeo and Flowers, 1986).

There has been reasonable consensus on what to target for genetic engineering of salt tolerance. These focus on ion transport and compartmentation, synthesis of compatible solutes (osmolyte or osmoprotectant), oxidative protection, and metabolic processes that are ‘weak links’ (Bartels and Nelson, 1994; Murghia et al., 1995; Bohnert and Jensen, 1996; Bohnert and Shen, 1998). Ion transport and osmolytes are obvious candidates given the prominence attached to the regulation of ion transport and compartmentation (see earlier reviews such as Flowers et al., 1977, 1986; Greenway and Munns, 1980; Yeo, 1983; Munns and Termaat, 1986). Attention has been given mostly to the products of structural genes (transport proteins, ion channels, enzymes of solute synthesis) with some attention to products that may have a regulatory role. This is not a general or encyclopaedic review. I concentrate upon what is known (or not known!) about processes commonly advocated as targets for intervention at the molecular level and discuss how this needs to be integrated with whole-plant physiology and eventually with plant breeding.

**Transport of ions**

Channels conduct ions across membranes according to the difference in electrochemical potential. Channels possess selectivity (between anions and cations and between different anions and cations). Their capacity for ion movement is controlled largely by gating (the proportion of the time during which they are open or shut). This may depend upon voltage, allosteric control or other (such as calcium-calmodulin) signals. Since selectivity and some aspects of gating can be regarded as properties of the structural gene product these have been seen as top candidates for manipulation. The same is true for transporters. These can move ions against their electrochemical potential difference across the membrane by primary active processes or by linkage to the gradient of another ion. Whilst there are also regulatory aspects, again many properties could be those of the structural gene product. One factor that has received attention are carriers that mediate sodium extrusion and for which bacteria and yeasts have been the popular model systems.

**Sodium extrusion**

In the yeast *Saccharomyces cerevisiae*, ENA1 (equivalent to PMR2) encodes the P-type ATPase suggested to be involved in sodium extrusion. It is regulated by the products of the genes HAL1 and HAL3 and by calcineurin. Calcineurin is a calcium-dependent protein phos-
that can result in the efflux of sodium. SOD2 codes the sodium/proton antiporter from *Schizosaccharomyces pombe* and has a homologue Z-SOD2 in the salt-tolerant *Zygosaccharomyces rouxii*, in which its expression is constitutive and independent of NaCl-shock; the predicted amino acid sequence of Z-Sod2p is longer than Sod2p and includes an extra-hydrophilic stretch in the C-terminal region (Watanabe et al., 1995).

The argument for transforming higher plants with structural or regulatory sequences that might confer sodium extrusion is presumably that this will counter intracellular sodium toxicity. While this may be so, the consequences of a plant cell doing this are complex and discussed below.

**Potassium : sodium selectivity**

As well as dealing with sodium, the cell must also acquire nutrient potassium. ‘Low-affinity’ potassium uptake by roots is believed to involve inward-rectifying potassium channels, allowing potassium to enter along an electrochemical gradient when potassium in the soil is relatively abundant (Smart et al., 1996). A collection of inward-rectifying potassium channels (KAT1, AKT1, KST1) have been identified from *Arabidopsis* and *Solomonum* from cDNA libraries complementing yeast mutants lacking endogenous potassium transporters and permit low-affinity (200 μM external potassium) growth (Smith et al., 1996): KAT1 and AKT1 have their highest homology near the N-terminus which contains the highly-conserved potassium-selective pore region and a voltage-sensing region.

KAT1 probably does not mediate potassium uptake by roots as it is expressed predominantly in leaf tissue, in leaf guard cells and vascular tissue of stem and root (Nakamura et al., 1995), and has been proposed as a mechanism of low-affinity uptake into guard cells. KST1 (S for *Solomonum*) from potato is also localized predominantly in guard cells and likely to be a functional homologue of KAT1.

HKT1 is a cDNA for a membrane protein from wheat roots whose properties correspond with the classical ‘high-affinity’ K-transport (channels can account for low-affinity). It functions by potassium/proton symport and its expression in both roots and leaves is correlated with sodium toxicity when potassium uptake is likely to occur (Schachtman and Schroeder, 1994). At toxic (mM) activities of sodium, HKT1 mediates low-affinity sodium-uptake while potassium-uptake is blocked (Gassman et al., 1996). Sodium transport by and sodium/potassium competition in HKT1 correlate with sodium toxicity and point mutations in the sixth putative transmembrane domain have been reported to increase sodium tolerance (Rubio et al., 1995). The mechanism of HKT1 from *Arabidopsis* appears to be 1:1 potassium/proton symport (Maathuis and Sanders, 1994) in agreement with Schachtman and Schroeder (1994).

There is however limited knowledge of how channels/transporters regulate intracellular potassium levels (Smart et al., 1996). If a local increase in calcium close to cell membranes and capable of modulating a variety of ion channels is a universal feature of ABA-response then calcium-coupled mechanisms exist that can account for inhibition of the plasma membrane inward-potassium-channel, activation of the plasma membrane slow anion channel, activation of the tonoplast potassium-selective and slow vacuolar (SV) channels: if calcium is not the second messenger then the signalling chains ‘have hardly begun to be understood’ (MacRobbie, 1997). So far as low-affinity transport is concerned, the suggestion is that channel activity rather than gene expression is regulated. A beta-subunit was recently cloned in mammals and plants (Tang et al., 1995) and this alters the characteristics of the channel. Salt tolerance will have a large element of maintaining cytoplasmic ionic homeostasis under conditions of adversity. It would appear vital to understand how conditions in the cytoplasm are sensed and used to regulate membrane transport processes. It is particularly pertinent when considering gene transfer to ask to what extent regulation resides at the level of the structural protein (eg allosteric) and to what extent different gene products are involved (whether in regulating expression of the gene or the activity of the transcript).

Locating the culprit for sodium influx in higher plants has so far proved somewhat elusive. Modification of sodium influx (essentially “blocking a leak”) may prove more energy-efficient that letting sodium in and then pumping it out again, so it is possibly the preferable alternative to engineering sodium efflux.

Hexaploid wheats (AABBDD genome) accumulate less sodium and more potassium in expanding and young leaves than tetraploid (AABB) wheats. This ‘K/Na discrimination factor’ has been recognized in the D-genome Triticeae in a long series of investigations (Gorham et al., 1986) has been localized to the 4D chromosome through the use of chromosome substitution lines in which B-genome chromosomes are replaced singly by their D-genome homoeologues (Gorham et al., 1987). Recent evidence points to the discrimination factor being a single locus (KNA1) and was mapped both as a qualitative and as a quantitative trait to the 4DL arm (Dubcovsky et al., 1996).

Examination has been made of whether leakage of sodium through potassium channels could account for differences in potassium/sodium discrimination. Patch-clamp studies in wheat and other glycophytes have found little sodium permeation through voltage-gated potassium channels (Schachtman et al., 1991; Gassmann and Schroeder, 1994; Tyerman et al., 1997).

SpTRK encodes a potassium-specific transporter in
Schizosaccharomyces pombe (hence the prefix Sp) and was located by cDNA complementation of trk (i.e. deficient expression of TRK) mutants of S. cerevisiae and shows high homology to TRK1 and TRK2 as well as HKT1 (from wheat). Patch-clamp analysis showed an inward potassium current in complemented cells and this showed proton activation (implying symport), but no inward current when potassium was substituted by sodium (Lichtenbergfratie et al., 1996).

Other wheat plasma membrane transporters with sodium transport capability, such as HKT1 (Rubio et al., 1995) and the calcium-selective, voltage-dependent rca channel (Pineros and Tester, 1995, 1997) have not been demonstrated to have characteristics consistent with those of the processes underlying sodium uptake by intact roots. They are sensitive to higher concentrations of calcium than intact roots and are voltage-dependent, whereas sodium influx appears to be voltage-insensitive in intact roots.

Membrane-potential-dependent uptake of sodium and potassium antiport by plasma membrane vesicles were measured in a tetraploid (AABB) and a hexaploid (AABBDD) wheat: inhibition of sodium influx by calcium was greater in the hexaploid, but overall it was concluded that neither of the measured transport processes was responsible for the discrimination trait on 4DL (Allen et al., 1995).

KNA1 does not seem to fit the properties of any known channel or carrier. However, it could be regulatory rather than structural. Discrimination has also been thought to reside at ion release to the xylem and so could involve a tissue-specific character that has yet to be recognized.

Several papers now describe cation channels that show poor overall selectivity and which are permeable to sodium (Amtmann et al., 1997; Roberts and Tester, 1997; Tyerman et al., 1997) which are therefore potential candidates for sodium entry in saline conditions. These channels are inhibited by extracellular calcium at similar concentrations that inhibit sodium influx (Davenport et al., 1997) and attempts are being made to characterize them using planar lipid bilayers (R.J. Davenport and M. Tester, personal communication). If a non-selective cation channel is a pathway of sodium entry in saline conditions then its down-regulation would enhance salinity tolerance, but as the function of this channel in non-saline conditions may be to facilitate the entry of a nutrient then this will have complicated consequences (M Tester: personal communication). Improving selectivity might be a better, though more difficult, task than down-regulation.

Transport in the whole-plant context

A further complication in all this is that different higher plant species differ in the underlying mechanism of sodium influx to the plant, and that this is not necessarily via carriers or channels. In rice, belonging to an aquatic genus with limited control of water loss (Yeo et al., 1997), apoplastic leakage pathways, considered to be related to root anatomical development, account for heritable differences in sodium transport (Yeo et al., 1987; Yadav et al., 1996; Garcia et al., 1997b). In this species, membrane-based potassium:sodium selectivity is masked by a membrane-independent parallel pathway. It has recently been shown that this leakage pathway was of minor importance in both a salt-tolerant and a salt-sensitive cultivar of wheat (Garcia et al., 1997b). This has two implications. Firstly, that cell-based processes can become subordinate to processes based at higher levels of organization. Secondly, and due to the impact of higher levels of organization, that how higher plants will work in a given situation is not easily extrapolated from the behaviour of single cells.

A simple example is the question of sodium extrusion, which may be a sound option for a bacterium or a yeast in a solution. If a root extrudes sodium it is not going to go away, not in the soil at any rate, where the mass flow of solution is predominantly towards the root and much, much greater than diffusion away from it. There will be advection of salt towards the root because the mass flow of solution is partitioned at the root surface, the water being taken up and the salt left behind. Even in a species with substantial leakage (rice) this partitioning (or salt ‘exclusion’) is still 95% efficient and will be better in cases such as mangroves without salt glands. Extrusion would be against an ever-increasing gradient becoming more costly and more difficult. Extrusion of salt in the leaf (salt glands apart) would essentially be a suicidal act. ‘Outside’ the leaf cell is an apoplastic volume that comprises about 1% of the solute-available space in the leaf. A sodium ion in the apoplast is going to be about 100 times as damaging as a sodium ion in the protoplast, and this has been formalized in the ‘Oertli’ effect (Flowers and Yeo, 1986; Flowers et al., 1991). Putting sodium extrusion into higher plants is not completely without potential, but it would have to be under the control of tissue-specific promoters and/or regulatory systems that did not just make sodium extrusion ‘mostly’ in the root, but made sure that it never happened in the shoot. Even then, the value in a soil below field capacity is doubtful, though salt-extrusion can be predicted to work quite well in nutrient solution in the laboratory!

Weak links

Certain processes or individual enzymes may be especially sensitive to salinity and if they have key roles as gatekeepers or regulators of major pathways then they may have a disproportionate or ‘limiting factor’ impact upon metabolic sensitivity in particular organisms. The argument is that overcoming the sensitivity of such key
processes may have a noticeable effect on salt tolerance. One process that has received a lot of attention recently is that regulated by the yeast gene HAL2.

HAL2

HAL2 from yeast encodes a 3(2'),5'-bisphosphate nucleotidase (DPNase) which dephosphorylates PAP (3(2')-phosphoadenosine 5'-phosphate) and PAPS (adenosine 3'-phosphate 5'-phosphosulphate) which are intermediates in the sulphate assimilation pathway. This represents a 'futile sulphur cycle' involved in the regulation of the flux of sulphur from sulphate to cysteine or methionine (Peng and Verma, 1995; Murguia et al., 1995). The HAL2-encoded nucleotidase is inhibited by sodium and lithium but not by potassium and its inhibition by sodium resulted in PAP accumulation (whereas heat-shock and oxidative stresses did not); HAL2 overexpression attenuated PAP accumulation suggesting that HAL2-nucleotidase cation-sensitivity is a key determinant of salt-sensitivity in yeast (Murguia et al., 1996).

E. coli has a cysteine auxotrophic mutant (cysQ), met22 is a methionine auxotrophic (HAL2 mutant) in yeast (which cannot use sulphate, sulphite or sulphide but has wild-type activities for sulphate assimilation and sulphur uptake). The cDNA of RHL (a plant homologue of HAL2 cloned from rice) complemented cysQ and met22 (Peng and Verma, 1995). SAL1 from Arabidopsis (a cDNA from salt-stressed roots) homologous to HAL2 (yeast) and cysQ (E. coli) allowed a hal2/met22 mutant to grow on sulphate (Quintero et al., 1996). Expression increased intracellular lithium tolerance and modified sodium and lithium fluxes and it was proposed that the SAL1-product had both nucleotidase activity (sulphur metabolism) and phosphoinoside signalling activity (sodium and lithium fluxes) (Quintero et al., 1996). Sequence comparisons had revealed conserved motifs that relate the rice DPNPase (the product of RHL which is about the same as HAL2) to the inositol monophosphate family, borne out by cationic activation and sensitivities (Peng and Verma, 1995).

Protein synthesis

The whole process of protein synthesis was identified twenty years ago as a key 'weak link' (Weber et al., 1977; Wyn Jones et al., 1979; Gibson et al., 1984). In the halophilic Suaeda maritima there were modifications in the ionic requirements of protein synthesis particularly in respect of sodium substitution for potassium (Flowers and Dalmond, 1992) though the requirements remain demanding. The organization of higher plants offers more opportunity than in single-celled organisms. It is demonstrably possible to maintain completely different conditions in reproductive and meristematic regions than in mature tissue, conserving the resources to satisfy the more demanding processes at their main sites while allowing conditions elsewhere to be poorer.

Osmotic solutes: osmolytes and osmoprotectants

A generalization applicable to all organisms with the exception only of the halophilic archaea in long-term adaptation to hypersaline (as well as desiccation and freezing stresses) is that organic solutes, osmolytes, are central and elevated inorganic ion activities and/or reduced cell volume are tolerated at most in the short-term only (see the extensive review of Yancey, 1994). The term 'compatibility' was used for such organic solutes by Brown and Simpson (1972), highlighting the fact that (polyhydric alcohols in that case) were non-perturbing of metabolism at concentrations where sodium and chloride were disruptive. Compatible solutes have been defined as organic osmolytes responsible for osmotic balance and at the same time compatible with the cells' metabolism (Galinski, 1993). Furthermore, such compatibility is a general property of protein/solute interactions, irrespective of species, and irrespective of the nature of the stress. Osmolytes are a wide range of compounds. These may be sugars or polyols or more specialized compounds. Quaternary ammonium compounds and the functionally similar tertiary sulphonium compounds (for a full review see Rhodes and Hanson, 1993) contain fully methyl-substituted nitrogen or sulphur atoms, respectively, creating a permanent positive charge on the N or S moiety. Such zwitterionic compounds were developed early as compatible solutes, de novo synthesis being found in several halophilic methanogenic archaea (Lai et al., 1991). Within this group of compounds glycine betaine is an ubiquitous protein-stabilizing osmolyte occurring from bacteria to higher plants and animals. In addition to osmoregulation it stabilizes the oxygen-evolving activity of photosystem II protein complexes by protecting against the dissociation of the regulatory extrinsic proteins and also stabilizes the manganese cluster. The ability to stabilize macromolecules under dehydration stresses as well as conferring thermal stability gives rise to their synonym of 'osmoprotectant' (Yancey, 1994). This implies that these compounds are directly beneficial to the stability of macromolecules and not only indirectly through providing an acceptable solute at osmotically effective concentrations. They are strong water-structure formers and probably excluded from the hydration shells of proteins accounting for their role as stabilizers of the hydration shells of native proteins (Galinski, 1993; Rhodes and Hanson, 1993; Papageorgiou and Murata, 1995). A distinction is made between osmolytes that are osmoprotectant (such as glycine betaine) and those that are radical scavengers (such as mannitol) and so protect against oxidative damage (Bohnert and Jensen, 1996b; Bohnert and Shen, 1998). An examination of stress
Glycine betaine

Glycine betaine accumulates in many species of the Poaceae and Chenopodiaceae (see Flowers et al., 1986, and references therein) but is absent from many crop species (eg rice) and the model species in plant transformation, *N. tabacum*. There has therefore been considerable work on engineering the production of glycine betaine in species that do not produce it naturally.

*E. coli* has a cluster of bet genes (*betA*: choline dehydrogenase, *betB*: betaine aldehyde dehydrogenase, *bet1*: a putative regulatory protein and *betT*: the choline transport system). A freshwater cyanobacterium was transformed with the bet cluster and expression detected by northern analysis and the detection of glycine betaine (Nomura et al., 1995).

In higher plants the pathway to glycine betaine synthesis is short and straightforward: choline monooxygenase (CMO) converts choline to betaine aldehyde and betaine aldehyde dehydrogenase (*BADH*) converts this to glycine betaine (spinach, Burnet et al., 1995). CMO is thought to be an iron-sulphur protein which in its native form is a homodimer (Burnet et al., 1995). In sugarbeet the BADH activity increased 2–4-fold in leaves and roots as NaCl was increased from 0 to 500 mM, the increase in BADH activity being correlated with the level of translatable BADH mRNA. There were at least two copies of BADH in the haploid sugar beet genome and analysis of cDNA clones showed small nucleotide sequence differences consistent with the existence of two different BADH alleles (McCue and Hanson, 1992).

Many species including tobacco lack both CMO and BADH. Spinach and sugarbeet cDNA sequences encoding BADH were expressed in tobacco and, even without a typical transit peptide, BADH was still targeted to the chloroplast in the leaves of transgenic plants. Expressed levels and substrate affinity was comparable with the native enzyme and transgenic plants were able to synthesize glycine betaine from supplied betaine aldehyde showing a constitutive ability to transport betaine aldehyde into the chloroplast. The glycine betaine so synthesized was not metabolized further and accumulated to concentrations similar to those plants that accumulate it naturally (though it is important to know that there is no information as yet on its subcellular localization in transgenic plants). Betaine aldehyde is toxic when supplied exogenously in tobacco unless it was transgenic (Rathinasabapathi et al., 1994). A BADH cDNA was cloned from barley: an open reading frame encoded a protein with high homology to BADH enzymes from the Chenopodiaceae and *E. coli*. Transgenic tobacco expressed the BADH protein and its enzymatic activity. There were 8-fold increases in BADH mRNA levels in leaves of barley under high-salt conditions. Levels were maintained under sustained stress, but decreased when stress was removed. Accumulation of BADH transcripts was a common response to osmotic stress however caused (Ishitani et al., 1995).

Not only is the pathway straightforward, but the genetics of betaine accumulation also appears to be simple. Near-isogenic pairs of maize lines differ for alternative alleles at a single locus. The wild-type *Bet1* makes glycine betaine and the recessive deficient mutant *bet1* does not. Glycine betaine-deficiency in maize was associated with a significant increase in the free choline (precursor) pool, but the increase was not equivalent to the decrease in the glycine betaine pool. There was also an increase in the serine pool (precursor to choline) suggesting that choline must down-regulate its own synthesis (Yang et al., 1995). Glycine betaine-deficient lines of *Sorghum* have also been identified. In crosses between glycine betaine sufficient and deficient mutants the ratio in F2 was 1:2:1 and back-crosses to deficient parents segregated 1:1 consistent with single-gene inheritance (primarily additive gene action) (Grote et al., 1994).

Trehalose

There are numerous examples of osmolytes other than glycine betaine (see the reviews of Rhodes and Hanson (1993); Yancey (1994) for example) and there is no virtue in cataloguing them here. One that needs some mention is trehalose, because of the suggestion that it has more than one role.

TPS1 from *Saccharomyces cerevisiae* encodes trehalose-6-phosphate synthase, which is regarded as both a metabolic enzyme and a regulator (Serrano et al., 1998) because TPS1 has been reported positively to modulate the heat-shock response as well (Hazell et al., 1995). Serrano et al. (1998) suggest that the products encoded by HAL1 (see above) and TPS1 may have a generalized role in activating stress-defence systems. The homologous CIF1 affects the transcriptional responses of many osmotically-induced genes including ENA1 (Hazell et al., 1977). The regulatory aspect devolves upon the mechanism of regulation of the sugar-phosphate pool by trehalose-6-phosphate synthase which is a futile cycle of trehalose synthesis and hydrolysis, analogous in some ways to the futile sulphur cycle.
Tobacco, transgenic for trehalose synthesis, was reported to have improved drought and salt tolerance (Romero et al., 1997), but there was also a changed carbohydrate profile suggesting changes in basic biochemical pathways and the resultant average tissue concentration of trehalose (<0.5 mM) appeared too low to be performing a conventional osmoprotectant role. Plants transgenic for trehalose synthesis also showed linked severe morphological alterations including stunted growth. The morphological changes (it is no simple matter to disentangle effects on stress tolerance from consequential effects of changes in growth) make interpretation difficult at the moment.

Relation of glycine betaine production and plant tolerance

Despite the apparent biochemical and genetical simplicity of the glycine betaine pathway and the success at transformation and expression, this has resulted in little if any benefit for plants in the field (Bohnert and Jensen, 1996b; Bohnert and Shen, 1998; Flowers et al., 1998).

The reasons for this may be many, and cover regulation, compartmentation of the solute and the cost of its production. There is also the more general consideration that, for salinity at least, the ability to produce glycine betaine is only likely to benefit the whole plant as part of the overall intracellular compartmentation ‘package’. This means that not only the compartmentation of glycine betaine must be regulated, but that of inorganic ions as well. Also, unless the total flux of inorganic ions into the plant is suitable, any potential benefit at the cellular level may be masked either by inadequate osmotic adjustment or by ion toxicity in the plant as a whole.

Compartmentation

In higher plants that are adapted to use glycine betaine, it is compartmentalized in the cytoplasm (Hall et al., 1978). The cytoplasmic volume fraction in succulent halophytes is quite small (Hajibagheri et al., 1984) and even if glycine betaine is accumulated to concentrations that can balance seawater the absolute quantity involved is still relatively minor. This is important because each molecule of glycine betaine contains five carbons and a nitrogen. If there was no compartmentation at all then the halophyte *Suaeda maritima* would need to accumulate about 800 mg of glycine betaine (about 400 mg of fixed carbon) for every gramme of dry weight, i.e. the plant would be committing around half of its net assimilation to glycine betaine production. This would be an enormous yield penalty in an agricultural context—and does not appear to have been even ecologically viable in higher plants—because the succulent halophytes do compartmentalize glycine betaine in the cytoplasm. This raises the question of what a transgenic plant will do with glycine betaine (or any other osmolytes) if given simply the capacity to synthesize it. Unless recipient plants have constitutively the capacity to compartmentalize and regulate the production of osmolytes in response to osmotic stress, then it will be necessary to supply more than the capacity to make choline monoxygenase and/or betaine aldehyde dehydrogenase. As remarked earlier, the techniques for subcellular localization of glycine betaine have long been available and it would be very informative to know where the compound is localized in transgenic plants.

Not only the osmolyte but inorganic ions also need to be compartmentalized if the overall package is to work. There have been considerable recent advances in the molecular and functional characterization of the two primary active transport mechanisms developing the electrochemical potential difference in protons at the tonoplast, summarized in the physiological context by the review of Barkla and Pantoja (1996). In sugarbeet a vacuolar proton-ATPase and a vacuolar sodium-proton antiporter contribute to compartmentalization of sodium in the vacuole. A partial cDNA of the proton-channel (subunit c) was used to monitor transcript levels. There was a large increase in the c-subunit mRNA following exposure of plants to 400 mM NaCl for 48 h. This was paralleled by increases in transcripts of the catalytic subunit (a) and of V-ATPase protein (Kirsch et al., 1996). All plants compartmentalize organic and inorganic solutes with considerable efficiency; the two or more orders of magnitude difference in proton concentration at the tonoplast being the most obvious example, but they do not all compartmentalize salt effectively. The halophyte *Suaeda maritima* does compartmentalize salt effectively, but the picture that emerges from studies of the tonoplast of this species does not point to any simple structural gene modifications. There were adaptations in the fatty acids and sterols that were all consistent with minimizing passive leakage, including the unusual preponderance of the planar sterol cholesterol, these were quantitative rather than qualitative (Leach et al., 1990). Proton-pumping capacity was similar to glycosphytes, and ion channels had the same characteristics combined with poor selectivity as tonoplast channels of non-halophytes (Maathuis et al., 1992). This leads to the conclusion that compartmentation of salt in the halophyte depends on regulation of permeability rather than structural targets for engineering. This suggests that we are looking at differences in the regulatory pathway: at perception, signalling or signal transduction. This, rather than structural genes for transport processes with different properties, may be the targets for understanding and manipulation in the future.

The recent elucidation of a class of membrane proteins termed aquaporins hints at the existence of some possibility for intracellular compartmentation of water.
Aquaporins are members of the MIP (membrane intrinsic protein) family and include TIP (tonoplast intrinsic protein) and PIP (plasma membrane intrinsic protein) members. Long suspected but only recently characterized (reviewed by Maurel, 1997) they are pore-forming proteins that conduct water molecules across membranes. The low activation energy points to channel activity and it has been assumed that the pore diameter is such that water molecules would pass in a single file, that is the pore diameter will exclude essentially everything else and contrasts with ion channels that also conduct water (Maurel, 1997). There is one report that salt stress down-regulates aquaporins in *Mesembryanthemum crystallinum* (Yamada et al., 1995). If aquaporins dominate transmembrane water movement and can be regulated as other channels then this introduces the possibility of control of water movement far beyond the general acceptance that it is the passive slave of water potential differences. There are enormous driving forces, for example, for sodium influx into cells in saline environments (a combination of membrane potential and concentration differences) but gating of channels can prevent this from becoming an inward flood. The implication is that gating of water channels could have an impact on intercompartmental movement of water.

**Perception of ions and ion concentrations**

Regulation of cytoplasmic ionic activity in higher plants in saline growth conditions is about compartmentation. This concerns transport process and their regulation at the plasma membrane and at the tonoplast. This in turn includes the selectivity and gating of channels conducting ions according to their free energy differences across the membrane, to active transport processes moving ions against their free energy differences, and passive or unregulated leakages. Regulation of cytoplasmic activities at different setpoints to those in the vacuole or apoplast implies concomitant regulation of other osmotically active (necessarily compatible and probably osmoprotectant) solutes to balance the uneven distribution of ions. Leaving aside the questions of plant ion uptake and water use, and looking in isolation at this one key cellular-based adaptation essential to permit a fundamentally salt-sensitive metabolism to operate in a plant in a saline environment, it is clear that a lot of factors have got to work, and work in concerted action, to achieve tolerance. One of the major gaps in knowledge is over how cytoplasmic ion concentrations are regulated. Given the plethora of channels and carriers it is hard to see how any one can confer salt tolerance. An obvious case would be if a deficiency in one was a major weakness.

In a salt-tolerant plant all these membrane transport processes are somehow co-ordinated to maintain the needed conditions. A halophyte has its vacuoles, 90% or more of a leaf cell, filled with 500–1000 mM NaCl, while keeping the cytoplasm at 150 mM or less. It is inconceivable to the author that this can be achieved without some sensing and signalling system that is able to measure the activities of sodium and potassium (and chloride) and regulate transporters of channels accordingly. There is little knowledge of how this is achieved. However, alterations in the sensing/signalling pathway might have further-reaching effects than alteration of individual structural components. The fairly specific requirements of processes such as protein synthesis indicates that the facility to recognize the activities of sodium and potassium independently and within sufficiently tight margins to achieve cytosolic homeostasis, does exist.

There are many specific examples of sequences and motifs that could be informative about ion recognition and many of these highlight the power of site-directed mutation as an experimental tool.

Alteration of a single glycine to alanine by site-directed mutagenesis had dramatic effects upon the activity of fructose-1,6-biphosphatase (pig): glycine at position 122 was essential for Mg2+ co-operativity and important in binding Mg2+ as well as for enzyme activity (Zhang et al., 1995).

A motif (Asp884-Asp885-Arg886-Trp887) (DDRW) is found in an extracellular peptide of the alpha-subunit of the animal plasma membrane sodium pump between two membrane-spanning regions (M7 and M8) and is similar to the structure (Gln399-Asp400-Cys401-Trp402) (QDCW) in the P-loop (pore-forming loop) of sodium and potassium channels (Fiedler and Scheiner-Bobis, 1996; Schneider and Scheiner-Bobis, 1997). The P-loop formed by the hydrophilic extracellular part of the channel protein is proposed to invaginate into the bilayer surrounded by the hydrophobic membrane spans of the protein. This is a possible basis for the control of ion fluxes through the membrane-spanning protein and bears similarities to a mechanism proposed for the structure of aquaporin.

Single mutation of the aspartates, particularly Asp885, to arginine considerably influenced enzyme-sodium interactions with the conclusion that Asp885 and Asp888 participate in sodium-ion pumping (Schneider and Scheiner-Bobis, 1997).

Several enzymes (inositol polyphosphate 1-phosphatase, inositol monophosphate phosphatase and fructose 1,6-biphosphatase) share the common motif (Asp-Pro-Ile or Leu)-Asp-(Gly or Ser) (DP(I/L)DG(S)/T/S) that has been shown to bind metal ions and to participate in catalysis (York et al., 1995). Common core structures for these proteins emerged when they were aligned according to this motif. The motif also has been found to be conserved in other related proteins including the products of the bacterial cysQ and yeast met22 (= hal2) genes. All have a common core structure diverged from a common ancestral protein and identical metal-dependent, lithium-
sensitive catalytic mechanisms (York et al., 1995). A mechanistic account for the salt-sensitivity of HAL2 (see ‘weak links’ above) is emerging.

Site-directed mutagenesis of a conserved region in neurotransmitter transporters identified Glu101 of the sodium- and chloride-coupled γ-amino-butyric acid transporter GAT1 as critical: mutation left 1% or less of activity and transient sodium currents were not observed (Keshet et al., 1995). An highly-conserved aspartate was demonstrated, again by site-directed mutagenesis, to be essential for the sodium-activation of the chorionagadotrophin receptor (Quintana et al., 1993).

An alignment of five amino acids is common to several sodium symporter carriers and was proposed as the putative sodium-binding motif (Deguchi et al., 1990). Site-specific mutagenesis was used to change arginine376 of the E. coli proline-sodium-symporter to lysine, glutamine or glutamic acid (Yamato et al., 1994) with the conclusion that the motif was not essential to sodium-binding though probably relevant to the structure of the symporter. Binding of cations to DNA has been measured showing selectivity for univalent cations as K > Na > Cs (Deng and Braunlin, 1996).

There may as yet be few definitive clues to binding motifs, but this would seem a very promising line of investigation towards identifying the sensor part of regulatory pathways without which it would be difficult to account for ion compartmentation.

Promoters and specificity

A key problem already alluded to is specificity of expression. If a gene for a sodium-eﬄux pump was expressed and active in a leaf of a transgenic plant it would be lethal almost instantly. Very tight control of site and timing of expression would be essential for this type of intervention. The consequences of producing glycine betaine where and when it is not needed may not prove lethal, but as discussed earlier, the costs of doing so may outweigh any advantage in agricultural terms. However, there has been progress in identifying tissue-specific promoters and fusing them to reporter genes. This implies the need to search not only for genes but promoters and to transform with appropriate hybrid constructs.

The gene encoding osmotin is derived from the N. sylvestris parent of N. tabacum. In cell suspension culture the gene is activated transcriptionally by treatment with ABA. The cloned promoter was fused to a reporter gene and in transgenic plants the promoter was transcriptionally activated by ABA and ethylene. The osmotin promoter was more active in root than in leaf tissue (Nelson et al., 1992).

AtPSR encodes pyrroline–5-carboxylate reductase, the final step in the biosynthesis of proline in Arabidopsis. By fusing the AtPSR promoter to a reporter, strong activity was demonstrated in a wide range of tissue locations that were either undergoing cell division or changes in osmotic potential, consistent with hypothesized roles in nutrient supply and dehydration response. Replacing the promoter with 5’-deletions implicated a 69 bp promoter region in the tissue-speciﬁc expression of AtPSR (Hua et al., 1997).

ABA-responsive proteins were examined in rice varieties differing in overall tolerance of salinity. Classic non-dwarf donors showed a greater ABA induction in response to osmotic shock and a greater range of ABA-induced proteins than a salt-sensitive variety (Moons et al., 1995). It is, however, too easy to confuse osmotic shock and salinity responses.

Several genes induced by salt in salt-tolerant alfalfa callus were cloned, although these were not induced by salt stress in the parent salt-sensitive cell-line. Two transcripts were similarly salt-induced in callus, but in plants tissue-speciﬁc expression can be seen (Winicov and Shirzadegan, 1997).

SalT is a cDNA clone containing an open reading frame coding a 145 amino acid protein whose mRNA accumulates rapidly showing organ-speciﬁc expression (sheaths and roots of mature plants and seedlings) in response to a variety of salt, osmotic and desiccation stresses and the exogenous application of ABA (Claes et al., 1990). The organ-speciﬁc expression first appeared correlatable with the pattern of sodium accumulation, but more recent results showed that organ-speciﬁcity was also determined by other factors, since SalT was expressed only in leaf sheaths of rice even though the salt concentrations in the leaf lamina were equivalent (Garcia et al., 1997a).

The cDNA of an ABA-responsive gene isolated from suspension cultures of Solanum commersonii was 98% homologous with tobacco osmotin. Accumulation of the corresponding transcript was regulated by ABA, cold temperature and low water potential treatments. Cold-induced accumulation was suppressed by an inhibitor of ABA synthesis and was restored by exogenous ABA application. There was also organ-specific accumulation of the transcript in response to ABA or cold treatment (Zhu et al., 1993).

Fusing the promoter of ARSK1 (coding an osmotically-inducible protein with structural similarities to serine/threonine kinase in Arabidopsis) to a reporter showed root-speciﬁc expression that was conﬁned outside the endodermis and maximal near the root apex (Hwang and Goodman, 1995). Fusion of the E. coli gene betB coding betaine aldehyde dehydrogenase to the promoter of ars1a encoding the small subunit of Rubisco in A. thaliana including the N-terminal transit peptide for chloroplast targeting resulted in betB being directed to the chloroplast in transgenic N. tabacum (Holmstrom et al., 1994).

Suitable constructs may therefore be possible to get transport genes expressed in the right place.
Practical advance: handling this information in plant breeding

There has not yet appeared any single, simple answer to salt tolerance. If there had been then it would have been expected that plant breeders would have solved this problem without input (or interference!) from plant scientists and more recently molecular biologists. The large amount of research into salt tolerance, commonly with practical crop improvement as its declared goal, has had disappointingly little success at developing salt-tolerant cultivars (Flowers and Yeo, 1995). It is possible that the requirements to deal with environmental stresses lie at a level of complexity, or of organization, that has so far confounded conventional and non-conventional approaches at crop improvement. It is becoming necessary to postulate the need to introduce more and more genes, combined with the appropriate promoters, targeting sequences and maybe control genes as well. And unless the resultant can be used as a donor in conventional breeding, then the whole process needs to be repeated many times, because it is now clearly recognized that stress-tolerance requires a large number of cultivars adapted to local conditions. Furthermore, most cultivars are short-lived. The practicalities of using a complex transgenic in conventional plant breeding are considerable.

Firstly the transformation event must be stably inherited. The limited success of cell culture selection to regenerate stable varieties, however often apparently salt-tolerant cell lines have been isolated, provides an important lesson about stability. The location of the transgene(s) in the genome will also matter. It will be of no practical use if the transgene(s) become linked closely with genes with undesired effects, hence the genetic analysis of many transformants will be needed. Transformation is essentially random, but for use as donor parents all must have the same genotype. Transformants need to be screened and a chosen transformant needs to be multiplied, which may be more laborious than making numerous transformants. But plant breeding could not cope with the same trait being linked to an advantageous trait on one chromosome in one parent and a disadvantageous trait on a different chromosome in another.

Transgenic plants are giving and will continue to give us information about how genes work that could not be conceived before this technology became available. However, if the development of salt-tolerant crops through genetic manipulation does involve a catalogue of genes and attendant control then use in crop improvement will be a quantum jump, not a trivial extension of this technology (as it may be in many genetically simpler situations).

But there is one puzzling factor that in a paradoxical way is a source of hope. Traits such as salt uptake and compartmentation are quantitative: they show continuous variation and cannot be classified as factors showing Mendelian inheritance. A methodology combining statistical methods with genome mapping has been developed to associate quantitatively variable phenotypes with qualitative genetic markers: quantitative trait locus (QTL) analysis (see review by Prioul et al., 1997). If the quantitative difference between halophytes and glycophytes was the sum total of small independent differences in all the bits and pieces needed to establish salt tolerance then these could reside scattered all over the genome. That salt tolerance had arisen independently in phylogenetically separate groups (Flowers et al., 1977) would then be surprising. Genetic analyses of quantitative traits seems to indicate manageable numbers of bits of chromosome having major effects upon some complex physiological traits (Prioul et al., 1997). Recently, root system morphological characteristics associated with sustained water acquisition under drought have been assigned to relatively few QTLs (Champoux et al., 1995; Yadav et al., 1997). Current results (M Koyama, A Garcia, R Koebner, T Flowers, A Yeo: unpublished data) are suggesting that a few QTLs may explain salt uptake in rice, but what is a QTL? Is it for instance a cluster of related genes or a regulatory gene with co-ordinating activity? This remains an open question.

Regulatory genes controlling photoperiod sensitivity and vernalization requirement are associated with constitutive differences (expressed in both non-saline and saline conditions) in shoot sodium concentration in the Triticeae: either these genes have pleiotropic effects upon response to salt or they are tightly linked to gene(s) that do so directly (Forster, 1994). As with dwarfing genes that affect salt concentration through vegetative vigour, it will be very difficult to unravel genetic from physiological linkage where genes with drastic effects on plant development are involved.

There are some reports of gene clustering in higher plants, that is genes involved in a phenomenon such as stress tolerance being sited in physical proximity (Forster, 1994). Cytogenetics offered the first possibility of introgressing whole or parts of chromosomes and considerably more sophisticated methodologies for gene transfer have developed and these are reviewed by Forster (1994). Addition, substitution or translocation of single chromosomes reduce, but still leave considerable, the introduction of alien genes, and although translocations have been successful, work focuses on transferring smaller and smaller segments of chromosome. A technique for introgressing short chromosome segments (so minimizing the insertion of other alien genes which may have undesirable effects on the agronomic genotype) are described by Luo et al. (1996). Fluorescence in situ hybridization is also proving invaluable in detecting the introgression
of alien chromosomes and chromosome fragments (Quarrie, 1996).

If a QTL is a regulatory gene then it may in the future be handled empirically via DNA-marker-assisted breeding or through finding out enough to handle it with the same technology as applied to the manipulation of structural genes.

Concluding remarks

The conclusion was drawn long ago that halophytes and glycophytes differed quantitatively rather than qualitatively and the extreme examples really lay on a continuum. With the exception of complex, multicellular salt-excreting glands there is no clear distinguishing feature of salt-tolerant plants. Their compartmentation is certainly better, their ion selectivity is probably better, their water use is lower and their water use efficiency possibly higher. Some key metabolic steps may be a little more tolerant. Even within each of these categories there is no obvious candidate gene for imparting an overriding change. An improvement may certainly be gained if the weakest link in a particular case is cured, but only until the next weakest link cuts in. Stress responses appear mostly to show quantitative inheritance. Quantitative trait analysis is providing cases where physiologically complex traits are governed by rather few quantitative trait loci. This gene showing organ-specific expression in response to salt stress possibly higher.

References


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