

BIOTECHNOLOGY FOR SALT TOLERANCE AND/OR ENHANCED WATER USE IN PLANTS – INTERESTING SCIENCE OR A PATHWAY TO THE FUTURE?

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ABSTRACT

The physiology and genetic basis of salt and other abiotic stress tolerances in plants is reviewed, and a practical definition for salt tolerance in the context of commercial crop production is explored. The parameters that define an envelope of conditions within which biotechnology is a logical and appropriate approach to plant improvement, and the ways in which biotechnology may complement and enhance conventional breeding methods are discussed. A selection of key biotechnology tools and their use in plant improvement for abiotic stress is reviewed. Progress with genetic improvement through manipulation of the key stress tolerance mechanisms in plants is reviewed, with reference to biotechnology approaches in a range of cultivated plant species. The review concludes with a discussion on the appropriate application of annual and perennial species in the context of long term sustainability.

Biotechnology has already contributed to the development of stress tolerant cultivated species and it is concluded that there is little doubt of further significant advances over the next decade. The appropriate deployment of these products to ensure long term sustainability is a question for agronomy and natural resource management, rather than biotechnology itself, requiring complementary land management to address the underlying causes and spread of salinity. Salt tolerant perennials with high water use, when managed appropriately, will have a particular role in contributing to rehabilitation of saline land and addressing the underlying problem of salinity, while contributing to increased production.

INTRODUCTION

Advances in biotechnology and genomics in particular have captured worldwide attention with achievements such as the sequencing of the human genome, and to a lesser extent, the genome of rice and the *Drosophila* of the plant world, *Arabidopsis*. Yet despite the massive global investment in plant biotechnology, delivery of products with tangible benefits into routine agricultural practice remain limited to a handful of crops, for a limited number of traits under simple genetic control. Notable examples include insect resistance in cotton, herbicide resistance in soybean and delayed ripening in tomato. Significant progress has also been made with disease resistance in a number of important crop species. Recently plants resistant to mercury have been successfully engineered using a bacterial mercuric ion reductase gene (Che *et al.*, 2003).

Despite the global significance of abiotic stress in limiting potential crop yields and, in particular, salinity which affects over one third of the world's irrigated land and significant

areas of dryland agriculture (Ghassemi *et al.*, 1995), and despite numerous promising reports in the literature, biotechnology has not yet delivered a single commercial release of a variety genetically engineered for abiotic stress tolerance. This is in part the result of the complex nature of stress tolerance in plants compared to the single gene traits that have been successfully manipulated to date. However, significant progress has been made in experimental trials, notably, the development of tomato plants that can grow, flower and fruit in the presence of 200mM (approx 18 dS/m) sodium chloride (Zhang and Blumwald, 2001). The achievements of Zhang and Blumwald, and those of many other researchers reported in the literature suggest it is inevitable that genetically modified crops for salt and stress tolerance will be commercially released within the next few years. The question then will be on the appropriate application and sustainability of crops grown from genetically tolerant varieties. In addition, genetic engineering is just one application of biotechnology to plant and crop improvement, and other areas have the potential to make a valuable contribution to improved agronomic utilisation of salt affected land. This paper will identify the situations where biotechnology is an appropriate approach to plant improvement for abiotic stress tolerance, review the potential for biotechnology to contribute to such genetic improvement, and explore the practical consequences of deploying salt tolerant plants from a variety of crop types.

GENETICS AND PHYSIOLOGY OF STRESS TOLERANCE IN PLANTS

Until recently, salt tolerance and tolerance to other abiotic stresses were believed to be complex, mutagenic traits (Allen *et al.*, 1994) and therefore difficult to control and improve by breeding. While tolerance to abiotic stress is still considered to be a complex trait, recent work has demonstrated the effect of major genes whose influence is modified by genes of smaller effect (Zhang and Blumwald, 2001). This understanding provides an important foundation to achieve improved stress tolerance, since directed breeding or genetic modification aimed at manipulating a small number of genes can be expected to achieve a large effect.

The control of abiotic stress by genes of major effect modified by many genes of small effect can be better understood by considering the mechanisms of salt and stress tolerance frequently employed by non-halophytic plant species. Excess soil salinity affects plants in two principle ways. Firstly, the high external concentration of salt creates an osmotic gradient making it harder for plants to extract water and nutrients from the soil at any given soil moisture level (Frommer *et al.*, 1999). In this manner, salinity exerts a similar physiological stress to drought. Osmotic stress may be the most important short-term effect of salinity on plants (Munns and Termaat, 1986). Secondly, sodium and chloride ions entering plant tissues may act through a specific toxicity or disruption of metabolic pathways as a result of ion imbalances (Allen *et al.*, 1994). Under conditions of long term exposure to salt, the maximum concentration of salt tolerated by fully expanded leaves is believed to be the most important factor affecting plant performance (Munns and Termaat, 1986).

Secondary effects of excess salt include increased expenditure of energy on maintenance respiration or ion transport, reduced energy for translocation of carbohydrates, and diversion of photosynthates from growth to osmoregulation (Allen *et al.* 1994). Also, the closure of stomata due to apparent drought stress induced by salt may reduce the availability of CO₂ for synthesis of carbohydrates while photosynthesis continues, leading to excess high energy free radicals which may directly damage plant cells.

Given the various mechanisms of plant responses to salinity, genes involved in salt tolerance include those controlling the following processes: (i) ability to exclude uptake of sodium and chloride into the root by active (ion channels) or passive (membrane permeability) means, but maintain selective uptake of ions important for plant growth; (ii) ability to compartmentalise sodium and chloride into the vacuoles of cells, or into senescent tissues, thus maintaining low cytoplasmic levels of these harmful ions, particularly in actively growing leaves; (iii) synthesis of osmoprotectants that help the plant to maintain cell turgor, to maintain water and nutrient uptake against an osmotic gradient created by salt in the soil, and to balance sodium and chloride compartmentalised in cell vacuoles or senescent tissues; and (iv) secondary stress responses that quench high energy free radicals to prevent cell damage. Genes controlling each of these processes have been identified and their potential for manipulation to enhance stress tolerance will be discussed later in this review. Avoidance mechanisms such as preferential root growth into relatively lower salinity pockets of soil, given soil is typically a heterogenous environment, may also play a role but are less well understood. While there is no direct genetic evidence for such a mechanism for salt tolerance, indirect evidence is displayed by *Lotus japonica*, where mutant genes have been shown to alter the ability of the root system to selectively exploit soil domains in response to rhizobia (Wopereis *et al.*, 2000).

PLANT IMPROVEMENT FOR SALT AND STRESS TOLERANCE

To achieve improvement in the salt and stress tolerance of plants, the objective must first be defined in order to provide a basis against which meaningful gains can be measured. Yokoi *et al.* (2001) identified the important elements of salinity tolerance as both the capacity to tolerate cellular hyperosmolarity and ion disequilibrium, while maintaining satisfactory yield. Mass and Hoffman (1977) integrated these concepts into a method of salt tolerance evaluation based on comparing yield at elevated salinities relative to non-saline controls (Figure 1).

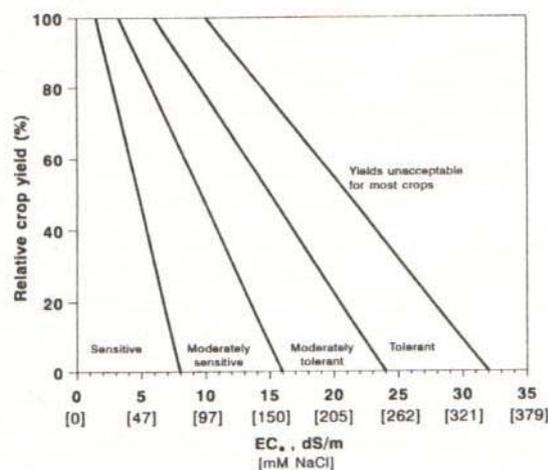


Figure 1: Divisions for classifying plant salt tolerance to salinity based on a comparison of yield at elevated salinity versus low salinity controls. Source: Mass and Hoffman 1977.

In this model, tolerance to salinity is effectively defined as capacity to maintain an acceptable yield within a practical working range of salinity. As such, practical salinity tolerance for crop production is a balance between yield and the physiological demand of dealing with salt stress. A plant that is capable of surviving extreme salinity has little value if the physiological demand of dealing with excess external and internal salt concentrations reduces growth and

yield below acceptable limits, particularly if the salinity level tolerated far exceeds the typical range to which it is are likely to be exposed in most field situations.

By evaluating yield at different levels of salinity, the classification proposed by Mass and Hoffman integrates survival and productivity over the full life cycle or crop rotation, and thus accounts for cumulative or long term effects of exposure to salt. However, the model is necessarily simplistic relative to the real world situation, where humidity, temperature, light, irrigation management, cultural practices, soil fertility, air pollution, soil calcium concentration, waterlogging, atmospheric CO₂ concentrations, soil type and the composition of soil salts themselves may all interact with salinity tolerance. Similarly, the nature of exposure to salinity may vary over a crop rotation. For example, the salinity level at different stages of plant development, and the rate of change of salinity in the root zone will both interact with plant tolerance. These considerations are important in any program to genetically improve salt and stress tolerance, regardless of the method employed, and highlight the inter-dependence of genetic improvement with management practices.

PARAMETERS INFLUENCING PLANT IMPROVEMENT FOR SALT AND STRESS TOLERANCE

Genetic improvement for any trait, regardless of the methods employed is explained by the fundamental plant breeding theory:

$$\Delta G = i \cdot h^2 \cdot \sigma_p / t$$

Where:

ΔG = response to selection or genetic gain per unit time

i = selection intensity

h^2 = heritability of the trait under selection

σ_p = the total phenotypic standard deviation in the breeding population; and

t = time.

This theory identifies the capacity to achieve genetic improvement for salt tolerance, quantifies the results that can be achieved for known values of each of the parameters, and can be used to guide the most appropriate strategy for genetic improvement. That is, it can be used to quantify the relative efficiency of conventional breeding and biotechnology:

- Selection intensity, i , is the proportion of a population that is selected as parents for crossing, and so relates principally to conventional breeding, but is also relevant to genetic marker guided breeding;
- Traits with low heritability, h^2 , are under limited genetic control and therefore difficult to improve by either conventional breeding or biotechnology, as environmental conditions play a significant role in the ultimate expression of the plant phenotype or form. Low heritability traits are typically controlled by many genes, each of small effect. Therefore, traits of low heritability such as yield are best improved by conventional breeding, where the entire genome is manipulated in each cycle of crossing. In contrast, biotechnology methods typically involve manipulation of single genes, or at best a small number of genes each of large effect, and use of biotechnology approaches is best suited to traits with high heritability. Recent developments in manipulation of genes controlling signal transduction pathways, where single genes control the expression of multiple other genes in biochemical pathways, may change this limitation of biotechnology in the future (Rai and

Srivastava, 2001). Notwithstanding, recognition that salt and stress tolerance in plants is under the control of major genes for a variety of physiological responses, the expression of which is modified by many genes each of minor effect, provides the necessary foundation to achieve improvement in stress tolerance through use of biotechnology.

- The amount of phenotypic variation, σ_p , in a population is a key determinant of the appropriate approach to genetic improvement. Where substantial phenotypic variation for a trait that is heritable exists in a population, then improvements can be achieved through conventional breeding and selection to combine favourable gene alleles into a commercial variety. However, conventional breeding and selection is imprecise given the expression of a trait such as salt tolerance in a parent selected for breeding is the net result of that individual's genes, the interaction between its specific package of genes, and its environment. Biotechnology may play an important role here. Where genes controlling a particular trait have been identified, then the precision of selecting parents for breeding, and in turn of selecting offspring that inherit these favourable genes, can be greatly improved by selecting both parents and offspring on their genotype as well as their phenotype. Dale and Chapparo (1996) showed that the efficiency of selection on genes or markers linked to genes, can triple the efficiency of parental selection for breeding for a low heritability trait ($h^2 = 0.025$) where just 20% of the additive genetic variation is explained by the tagged gene or linked markers. Where the proportion of additive genetic variation explained by the tagged genes or linked markers is greater than the heritability, selection on the genes or markers alone is more efficient than selection on the individual's phenotype (Lande and Thompson, 1990). A host of molecular biology techniques, together with genetic mapping (Lander and Botstein, 1989) and recent advances and availability of micro-array analysis (Wisman and Ohlrogge, 2000) have greatly improved the capacity to identify genes controlling plant traits and functions, and allow their manipulation where natural genetic variation exists.

The case for application of genetic engineering is most clearly evident where there is little or no genetic variation for a trait of interest in a population, in the species as a whole, or in related species that will interbreed. In the absence of phenotypic variation, then conventional breeding cannot achieve any improvement. The use of genetic engineering or protoplast fusion to introduce genes from one species into another species with which it would not normally breed, or the use of or mutagenesis and similar techniques to alter the base sequence of existing genes, are all methods of effectively introducing new variation into a species, thereby increasing σ_p and allowing improvements in the target trait to be achieved.

- Time between generations affects the rate at which useful plant improvement can be achieved and delivered into commercial practice. Conventional breeding is limited by the time for successive generations to germinate, grow to sexual maturity and set seed. Each cycle of breeding re-arranges gene combinations, and so introgression of a gene from one parent into a commercial variety may take many cycles of backcrossing to recover the original commercial variety with the added trait of interest. This process can be shortened where selection of useful genotypes can be made after the first generation of crossing, and commercially useful genotypes can be clonally propagated. Clonal propagation, itself an area of biotechnology, can be particularly valuable for species with long generation intervals such as trees (Dale, 2002).

Techniques such as genetic engineering, protoplast fusion and mutagenesis are less dependent on plant generation intervals and offer the potential to deliver genetic gain to

the field more rapidly than conventional breeding of many crop species. Genetic engineering and mutagenesis generally involve introduction or modification of a single gene or a small number of genes. When applied to an existing commercial variety, the package of genes that confer the variety's commercially valuable characteristics are largely unaltered with the exception of the introduced or modified gene or genes, and lines homozygous for the introduced or modified gene(s) can be recovered after a single cycle of breeding. Notwithstanding, the process of transformation, selection of transformed cells, regeneration of whole plants, screening transformants and crossing to produce homozygous lines can be lengthy, particularly in long-lived crops. However, techniques are improving and are being applied to an increasingly wider array of species (Rai and Srivastava, 2001), and it can be expected that improvements in the efficiency of this process will be achieved.

Perhaps one of the most severe limitations to delivery of commercial varieties of salt and stress tolerant plants to the field is not the technical process itself, but the legal, commercial and regulatory process of licensing useful genes and gaining approvals for commercial release of genetically modified organisms (GMO's). Many GMO's require use of technology from a number of sources (transformation techniques, promoter elements, selectable markers, etc, as well as the gene of interest), so require multiple licence negotiations for commercial use. Tomato plants genetically engineered to grow, flower and produce fruit at up to 200mM sodium chloride (Zhang and Blumwald, 2001), present one of the most promising developments in genetic engineering of crops for salt tolerance, but release of commercial varieties is expected to be at least four to five years away, being the time expected to complete technology licensing negotiations (Ed Blumwald, pers. comm.).

BIOTECHNOLOGY TOOLS FOR PLANT IMPROVEMENT

Although biotechnology is popularly perceived to be limited to genetic engineering involving transfer of genes across species boundaries or cloning of animals, it involves a broad suite of disciplines and techniques that can be applied to cellular and genetic mechanisms in plants. In its broadest sense, conventional breeding, which involves directed manipulation of genes by combining the genome of one individual with that of another using pollen as a vector, can be considered as a form of biotechnology.

Genetic mapping and marker assisted breeding

Genetic mapping involves correlation of segregating markers located throughout the plant genome, with expression of phenotypic traits. This correlation analysis is most readily carried out within a defined pedigree by comparing the suite of genetic markers carried by progeny that exhibit the trait of interest, with the suite of genetic markers carried by progeny not exhibiting the trait. This process allows the localisation, relative to nearby markers, of multiple genes affecting a trait of interest. By following the inheritance of markers, progeny from new crosses can be selected as juveniles based on their genotype. Numerous genetic marker systems have been developed including restriction fragment length polymorphisms (RFLP's) random amplified polymorphic DNA (RAPD's), simple sequence repeats (SSR's) or microsatellites; amplified fragment length polymorphisms (AFLP's); and sequence characterised amplified regions (SCAR's) (Henry, 1997). Most recent work is based upon the analysis of single nucleotide polymorphisms (SNPs).

Genetic mapping work to identify abiotic stress tolerance genes has been limited relative to work in gene discovery and transformation. Genomic regions or quantitative trait loci (QTL) have been identified for salt tolerance in the *Citrus grandis* x *Poncirus trifoliata* F1 hybrid and its backcross to *Citrus grandis* (Tozlu *et al.*, 1999), and for salt tolerance under waterlogging conditions in the *Eucalyptus camaldulensis* x *E. grandis* hybrid (Dale *et al.*, 1999), however, neither report has validated the QTL function in separate crosses. Where there is limited genetic variation within a species for stress tolerance, then genetic mapping will be most informative in hybrid crosses between interbreeding species where one of the parental species exhibits natural stress tolerance. Notwithstanding, genetic mapping may also be used to uncover useful cryptic genetic variation (QTL enhancing salt tolerance) within apparently non-stress tolerant species (Dale *et al.*, 1999).

Gene discovery, sequencing and microarray analysis

Most genes involved in salt and stress tolerance mechanisms discovered to date have been identified by “traditional” molecular biology techniques such as differential hybridisation, subtractive hybridisation etc, and by genetic data analysis of homologous genes from model organisms. Protein crystallography has enabled detailed investigation of the structure and function of proteins transcribed from salt tolerance genes, for example the high affinity potassium transporter gene (Doyle *et al.*, 1998). Recent progress in the capacity for both high throughput sequencing of expressed genes (cDNA’s) and nuclear genes has led to complete sequencing of the genomes of rice and *Arabidopsis* in a time period considered impossible in the early 1990’s. This development, together with microarray analysis where a copy of every gene from an organism can be deposited onto a microscope slide, now allows the profiling of gene expression across entire biochemical pathways to be analysed in a single set of experiments, providing a powerful tool for gene discovery and characterisation. A high level of gene sequence conservation between different species has accelerated the extension of gene discovery from model species. Boundaries to transfer of information for stress tolerance genes between species may not be limiting, with genes such as the high affinity potassium transporter from *Arabidopsis*, wheat (a monocot) and *Eucalyptus* (a dicot), sharing a high degree of homology at the protein level (Fairbairn *et al.*, 2000). More broadly, the genes expressed in resurrection plants encode polypeptides closely related to proteins abundantly produced during embryo maturation in seeds, one of the few structures in higher plants able to survive without water (Bartels and Nelson, 1994).

Plant transformation

Once genes involved in stress tolerance have been identified and their function confirmed in model systems, they may be employed to modify stress tolerance in crop plants, either as tagged genes in marker guided breeding when they occur within an interbreeding species, or by transfer across species boundaries through plant transformation.

Techniques for transferring foreign genes into plants include electroporation, polyethylene glycol mediated gene transfer; microinjection, particle or micro-projectile bombardment and *Agrobacterium*-mediated gene transfer (Holmberg and Bulow, 1998). These technologies are now available for many crops (Bartels and Nelson, 1994), and there are numerous examples in the literature reporting enhanced stress tolerance in crop plants expressing genes from each component step of the major known stress tolerance mechanisms. Specific examples are discussed in more detail under case studies.

Clonal propagation (tissue culture, cuttings, somatic embryogenesis)

Clonal propagation has moved from a major area of what was considered biotechnology research in the 1980s and early 1990's, but is now generally considered a more routine area of plant cultural research, even though there remain significant advances to be achieved in developing clonal systems for recalcitrant species, maximising multiplication rates, and in scale up of lab techniques to commercial practice.

Importantly, clonal propagation is almost a mandatory delivery system from laboratory modification to field deployment for outcrossing species, and species with long generation cycles such as trees. Clonal propagation can be used to support conventional breeding and selection programs as well as breeding programs guided by marker assisted selection. It may also be used as a delivery system for genetically modified species where it is impractical to generate homozygous inbred lines from transformed commercial varieties, or in situations where a gene is transformed into a donor line and subsequently introgressed by crossing into a commercial variety.

Clonal propagation by nursery cuttings has found practical commercial deployment in lines of *Eucalyptus camaldulensis* x *E. grandis* and *E. camaldulensis* x *E. globulus* hybrids selected for salt and drought tolerance (Dale, 2002). Techniques such as somatic embryogenesis and subsequent production of artificial seed offer higher multiplication rates and improved nursery handling practices relative to cuttings, but the development of this technique is presently limited to a restricted number of, mostly herbaceous, species.

POTENTIAL FOR PRACTICAL APPLICATION OF BIOTECHNOLOGY: CASE STUDIES

The application of biotechnology and, in particular, the use of genetic manipulation for plant improvement is a reality, with GM crops such as roundup resistant cotton and canola now under commercial cultivation. While field trials of crops genetically modified for tolerance to abiotic stress are limited, there is a large body of work reported in the literature, with progress made toward engineering stress tolerance across all the major biochemical processes.

Regulated ion uptake in salt tolerance

Most non-halophytic plants (glycophytes) respond adversely to excess sodium in plant tissues and, as such, modification of plants to exclude sodium is a logical target for genetic improvement.

Rubio *et al.* (1995) showed that one of the routes of sodium entry into the roots of salt stressed wheat plants was via the high affinity potassium-uptake transport (HKT) system, and that expression in yeast of the mutagenised HKT gene resulted in yeast that discriminated better than wild type against sodium. Rubio *et al.* (1999) subsequently demonstrated that one of several mutants of the wheat HKT gene reduced the co-uptake of sodium with potassium at low external sodium concentrations during cation uptake experiments in *Xenopus* oocytes, and reduced the blocking effect of sodium on potassium transport at high external sodium concentrations, thereby conferring increased sodium tolerance. While such work is in its early stages, these results indicate the potential to favourably manipulate this important characteristic. The identification and sequencing of genes for a range of other ion channels will also allow screening of breeding populations to identify naturally occurring variations

which may exhibit improved ability to exclude sodium and possibly chloride at the root-soil interface. Notwithstanding it has been postulated that use of an exclusion mechanism alone to engineer salt tolerance could result in drought stress unless the plant's internal osmotic potential can be maintained (Bohnert and Jensen, 1996), indicating the interdependence of stress tolerance mechanisms.

Compatible solutes

In response to dehydration stress caused by salinity, drought or cold, plant cells must adjust their internal osmotic potential to maintain equal water potential with the environment (Bartels and Nelson, 1994). This may be achieved by inorganic ions, but these become detrimental to cellular biochemistry at high concentrations, particularly in saline conditions where sodium, toxic to plants, may be the most abundant cation available, and must be sequestered into the vacuole (Bartels and Nelson, 1994). To maintain osmotic balance in the cytoplasm under such conditions, plants accumulate specific types of organic molecules known as compatible solutes. These compounds are also believed to play a role in stabilising membranes and/or macromolecular structures (Holmberg and Bulow, 1998). Genes coding for several relevant enzymes involved in terminal or near terminal steps in the synthesis of compatible solutes including as mannitol, sorbitol, proline, ononitol/pinitol and glycinebetaine have been cloned and used to transform a range of species resulting in improved salt tolerance.

A number of examples have been reported in the literature of plants engineered to over-express these compounds showing enhanced growth under salt or drought stress relative to controls. Examples using tobacco include: genes catalysing the synthesis of mannitol (Tarcynski *et al.*, 1993); ononitol (Vernon *et al.*, 1993); and proline (Kavi-Kishor *et al.*, 1995). In the case of the latter, the concentration of proline was below that expected to be solely due to osmotic adjustment, indicating the mechanism of proline action is not fully understood. Endogenous levels of glycinebetaine in plants are generally correlated with natural salt tolerance. Highly salt tolerant species such as *Distichlis* accumulate high levels, moderately tolerant species accumulate intermediate levels, and sensitive species accumulate low levels or no glycinebetaine (Bartels and Nelson, 1994). Tomato transformed with the BADH gene that catalyses the synthesis of glycinebetaine exhibited tolerance to salt, with plants growing normally at salt concentrations up to 120mM (approximately 11dS/m) (Jia *et al.*, 2002).

Compartmentalisation of sodium and chloride in cell vacuoles in salt tolerance

Perhaps one of the most promising reports on development of salt tolerant plants has been the expression of genes coding for the sodium/proton antiporter protein. This gene has been expressed in *Arabidopsis* (Apse *et al.*, 1999), tomato (Zhang and Blumwald, 2001), and more recently canola (Ed Blumwald, pers. comm.). *Arabidopsis* plants and tomatoes expressing the antiporter gene were able to grow, flower and produce seeds/fruit in the presence of 200mM sodium chloride (approximately 18dS/m). The antiporter protein acts to transport sodium into the vacuole of cells where it is compartmentalised, averting the deleterious effects of sodium in the cytoplasm, but maintaining osmotic balance by using the accumulated sodium (and chloride) to drive water into the cells. The transport of chloride into the vacuole is mediated by anion channels. Sodium continues to accumulate in the leaves until the capacity of the vacuoles becomes saturated. The leaves then die, which is also the strategy used by halophytes (Ed Blumwald, pers. comm.). As the antiporter is localised in the leaves, tomato

fruit displayed very low sodium. These transgenic tomato plants are now being tested in the field, but commercial release is still four to five years away.

Scavenging of free radicals in general stress tolerance

A secondary effect of salt, freezing and drought stress is the formation of reactive oxygen intermediates, or free radicals, as a consequence of photosynthesis continuing while stomata are closed, restricting the sink for these high energy molecules in the assimilation of CO₂ into carbohydrate. These high-energy oxygen intermediates damage membranes, membrane bound structures and macromolecules, especially in mitochondria (Holmberg and Bulow, 1998). When expressed in tobacco, genes encoding for the free radical scavenging compounds superoxide dismutase (SOD) and glutathione reductase (GR), derived from *E. coli* and rice respectively, exhibited less damage than controls when exposed to paraquat, a superoxide generating herbicide (Aono *et al.*, 1995). Similar results were obtained in a separate experiment, with transgenic tobacco plants in which SOD was targeted to the chloroplasts expressing a twofold reduction in leaf injury compared to the plants where SOD was targeted to the mitochondria, and a three fold reduction in damage relative to the control (Van Camp *et al.*, 1994). These results indicate the multiple physiological effects of abiotic stress, and the interdependence of plant response mechanisms that need consideration in any attempt to introduce stress tolerance into glycophytic crop plants.

Other approaches to achieving stress tolerance through biotechnology

Other approaches that have met with some success in building stress tolerance into plants include:

- Transformation with genes coding for the disaccharide, trehalose, found in resurrection plants, microbes and animals, which is believed to have a role as an osmoprotectant since it provides effective tolerance to desiccation at osmotically insignificant concentrations (Smirnoff, 1998);
- Manipulation of late embryogenesis abundance (LEA) proteins, found in seeds, which are one of the few structures in higher plants able to withstand extended periods without water (Bartels and Nelson, 1994). Over-expression of these genes in rice resulted in improved osmotic stress tolerance, delayed development of damage symptoms, and improved recovery after drought stress (Holmberg and Bulow, 1998). Interestingly, group 3 LEA proteins also share similarity with type 1 antifreeze proteins from certain arctic fish species (Holmberg and Bulow, 1998).
- Facilitation of oxygen diffusion to roots of waterlogged plants (a stress often associated with salinity) by the expression of bacterial haemoglobin genes in tobacco (Holmberg and Bulow, 1998).

Expression of transcription factors controlling a co-ordinated response of multiple stress pathways. As there is considerable overlap in the biochemical response to different abiotic stresses, and as there is cross-talk between stress signal transduction pathways, transcription factors promise the opportunity to achieve broad spectrum abiotic stress tolerance. This is illustrated by the induction of freezing tolerance in plants subjected to mild water stress, or to application of the plant growth hormone ABA (Lang *et al.*, 1994).

SUSTAINABLE DEPLOYMENT OF SALT TOLERANT PLANTS

In addition to salt tolerance and yield stability being important features of a salt tolerant crop as suggested by Yokoi (2002), a third factor, sustainability, needs to be considered for truly viable, stress tolerant cultivated plants. There is little doubt that biotechnology has vastly improved our understanding of the genetics, biochemistry and physiology of salt and stress tolerance in plants, and that techniques such as transformation will begin to deliver commercial varieties of an increasing number of stress tolerant cultivated species over the next decade.

In the case of cyclic stress factors such as drought and frost, where plants may be exposed to stresses for brief periods before returning to normal growing conditions, this will undoubtedly provide the potential for a significant boost to farmers faced with seasonally unpredictable conditions that may lead to periodic failure of annual crops, or significant yield reductions in perennial species such as horticultural crops and forest trees.

The situation is not so universally clear for salt tolerant species, where plants interact directly with the source of stress, and cropped paddocks are part of larger catchment processes where long term cropping sustainability relies on achieving water balance equilibrium at the catchment scale. In this situation, salt tolerant annual plants can do little more than buy time for continuing exploitation of land destined for continuing degradation if complementary action is not undertaken to address the underlying causes of salinity. Given their limited rate of water use relative to rainfall typical of salinised landscapes, annual crop species cannot contribute to a stabilisation of regional or catchment water balance necessary to halt the spread of salinity. As such, the extensive planting of such species will only contribute to the problem by allowing a continuation of the “do nothing” approach, while salinity levels continue to rise and soil resources degrade as a result of increasing sodicity, to the point that even salt tolerant species will no longer be able to grow and land must be abandoned. In this sense, the smart science of developing salt tolerant annual crops is blinkered to the practical realities of its application. This, however, is a question for agronomy and natural resource management rather than a defect in the products of biotechnology.

In contrast, salt tolerant perennial species, particularly those with deep root systems and high annual water-use, will have a valuable role in saline agriculture by providing the means to accelerate the rate of discharge from saline soils, while preventing excessive salt accumulation at the soil surface. Salt tolerant perennials with high water use planted on saline discharge sites alone are unlikely to occupy sufficient area to re-establish catchment water balance and halt the trend of advancing salinity. As such, they will need to be complemented by the parallel establishment of species with similar water use characteristics on non-saline, often drought-prone recharge areas of catchments. The total area planted to such species needs to be adequate to balance excess groundwater contributions, largely due to leakage past the rootzone of annual crops, that contribute to water-table rise. With appropriate management, salt tolerant perennial species offer the opportunity to rehabilitate saline land, provided conditions are provided for salts to be flushed back down the soil profile as the near-surface water-table is lowered.

Clearly, the sustainability of agricultural production systems using salt tolerant cultivated species is an equally important consideration to the technical challenge of achieving salt tolerance in naturally glycophytic species, and the future priorities for biotechnology should be set in appreciation of the practical application of the technical outcome.

CONCLUSIONS

Biotechnology is an appropriate approach to genetic improvement for stress tolerance in many cultivated plant species where: there is limited within-species phenotypic variation for stress tolerance; there are identifiable processes for tolerance of abiotic stress under the control of a small number of genes of major effect; and conventional approaches to breeding require long time periods to release of new varieties.

Disciplines of biotechnology such as clonal propagation have particular application to field delivery of outcrossing species and species with long generation intervals. Clonally propagated, salt tolerant eucalypts bred by conventional breeding are currently being deployed in Australia. Genetic mapping and gene discovery have potential to significantly improve the efficiency of conventional breeding approaches, particularly for low heritability traits, by improving the precision of parental and offspring selection. Gene discovery is also a central tool to identification of new sources of variation for use in genetic engineering.

Biotechnology has already made significant advances in improving our understanding of plant abiotic stress responses, and in altering a wide range of commercial crop plants to withstand abiotic stress. It will have an increasingly significant role as gene pathways activated in response to stress are characterised using techniques such as microarray analysis, key genes in these pathways, often with broad effect across different forms of stress and plant species, are cloned and experience with their expression in non-tolerant plant species is accumulated.

Salt tolerant plants developed through genetic engineering are already a reality, and it is expected that continuing progress in existing areas of investigation, together with anticipated advances in biotechnology itself, will lead to further significant advances, with commercial release of abiotic stress tolerant plant varieties expected within the next decade. Canola may potentially be the first genetically modified species with broad-acre application to areas affected by dryland salinity in Australia.

As with any technology, the use of the products must be appropriate. In the case of plants improved for stress tolerance, this is a question for agronomy and natural resource management, rather than biotechnology itself. Widespread deployment of salt tolerant annual crops without complementary action to address the underlying causes and spread of salinity will only buy time while land resources continue to degrade and eventually become irrecoverable. Salt tolerant perennials with high water use will have a particular role in contributing to rehabilitation of saline land and addressing the underlying problem of salinity, but will also require complementary supporting action to achieve the scale of groundwater control often required in situations of both dryland and irrigation salinity.

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