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REVIEW

# The role of mutant cell lines in studies on environmental stress tolerance: an assessment

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## Introduction

The theoretical benefits of selection of mutant cell lines, for contributing improved germplasm to breeding programmes, has led to a very strong emphasis on these applied objectives, while the use of such lines for fundamental investigations has generated relatively little attention. This is particularly true in the case of cell lines modified in their response to environmental stresses. Taking as an example salt tolerance, probably the most widely studied phenotype *in vitro*, the strategy adopted for selection in most recent reports differs little from that used to obtain the first lines many years ago (Dix and Street, 1975; Nabors *et al.*, 1975). It is simply being applied to an ever increasing range of species. In most cases, characterization of the tolerant phenotype is at best superficial, and sometimes non-existent. Fortunately there are sufficient exceptions to indicate the potential of basic studies on tolerant cell lines, and plants recovered from them, for providing insights into mechanisms of salt tolerance.

There are clearly two main approaches to unravelling the complex interactions between higher plants and adverse environmental factors. One is to carry out baseline studies on a single species with a well-defined stress response. Examples of the value of this approach include the identification of compatible solutes such as glycinebetaine in halophytes (Storey and Wyn Jones, 1975) and, more recently, molecular studies on the process of cold acclimation (Dunn *et al.*, 1990; Mohapatra *et al.*, 1989), and numerous other instances could be cited. The alternative, and equally valuable, approach is to undertake comparative investigations on species, varieties, or tissues, differing in their response to a stress. A classic study of this kind was that by Lyons and Raison (1970) which pointed to a phase change in biological membranes as the basis for chilling injury, through comparing respiratory activity in mitochondria isolated from chilling tolerant and sensitive species. Such comparative studies are particularly informative when performed on material with a narrow genetic base, as, for example, in comparisons between tomato, and its more salt-tolerant wild relatives (Tal *et al.*, 1978, 1979), and reach their ultimate utility when carried out on mutants differing only in their response to the stress in question. This is of course self evident, having long been the basis for elucidating metabolic and developmental pathways in organisms as diverse as bacteria, plants, *Drosophila*, and man. The question which then arises is the extent to which cell culture technology can provide mutants not readily selected at whole plant level, and what kind of information has been, or will be, obtained by analysing such mutants. An assessment of the achievements and potential in this area is the intention of this short review. Approaches and procedures for selection of mutant cell lines are not emphasized, except where recent innovations have led to important breakthroughs, or where successful adoption of indirect selection strategies has itself revealed information on the nature of stress tolerance.

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## Salt tolerance

That a capacity for improved salt tolerance exists within the genome of non-halophytes is strongly indicated by studies on whole plants. Warne and Hickok (1987) used the haploid gametophyte generation of the fern *Ceratopteris richardii*, to select for salt tolerance, and their subsequent genetic analysis provided convincing evidence for the involvement of a single nuclear gene. In crop plants it seems likely that differences in salinity tolerance can also be detected among individuals in cultivars of outbreeding species, or in segregating populations of inbreeders, but that these differences are governed by genes with additive effects (McNeilly, 1990). Comparative studies between plants differing in salinity tolerance, studied *in vitro* and *in vivo*, quickly suggested that there was frequently, but not always (McCoy, 1987), sufficient cellular basis for the differences in salinity tolerance, to justify *in vitro* selection as an approach to obtaining tolerant mutants. The fact that true mutants with improved tolerance have appeared very rarely probably attests to naivety of researchers in assum-

ing the selection pressure should be simple and straightforward. It appears that plant cells can quite readily undergo an epigenetic adaptation to increasing levels of salinity, and that this obscures the selection of rare mutants with true tolerance. Furthermore, selection may be complicated by the involvement of both water deficit, and specific ion stresses, and the value of separating these by prior osmotic adaptation has been suggested (Harms and Oertli, 1985).

The first convincing demonstration of stable, meiotically transmitted, salt tolerance, selected *in vitro*, was that by McHughen and Swarz (1984) on flax. Greenhouse tests on the resultant cultivar, Andro, however, revealed a general improvement in vigor leading McHughen (1987) to suggest improved seedling vigor, rather than specific salt tolerance, as the basis for its improved growth under saline conditions. Field tests at a number of salt-affected sites do, however, indicate a clearly improved salt tolerance during emergence of Andro over its parental variety (Rowland *et al.*, 1989). Andro could therefore be regarded as providing the first opportunity for examining the physiological and molecular basis of salinity tolerance in a mutant selected *in vitro*. To date, however, studies on it have been restricted to an investigation of cross tolerance to other stresses, (O'Connor *et al.*, 1991) which suggests improved thermotolerance but not frost tolerance and speculates about a possible role for abscisic acid (ABA) in the stress adaptation process.

After a further lag a recent spate of articles describes *in vitro* selection, plus reliable inheritance, for improved salt tolerance in rice (Vajrabhaya *et al.*, 1989), sugarbeet (Freytag *et al.*, 1990), *Brassica juncea* (Jain *et al.*, 1991; Kirti *et al.*, 1991) and *Nicotiana plumbaginifolia* (Sumaryati *et al.*, 1992). A reason for this success may be the use, in all cases except the last, of embryogenic cultures, or other organized cultures with a strong potential for regeneration through adventitious shoots or somatic embryos in contrast to the callus or cell suspension cultures more commonly used. The *N. plumbaginifolia* mutants were selected in haploid protoplast cultures. Protoplasts should be used more for this kind of work since, in addition to providing a single cell culture system, they are inevitably 'osmotically adapted' due to their cultural requirements, making them particularly suited to selecting for tolerance to specific ions. This report was also the only one to exploit a mutagenesis treatment (UV-irradiation) and the only one to demonstrate Mendelian inheritance of tolerance as a monogenic dominant trait. Furthermore, these workers used the same procedure to select mutants resistant to KCl and polyethylene glycol-induced water stress, as well as NaCl. These are precisely the kind of stable, genetically well characterized, mutants needed for investigations into mechanisms of stress tolerance. They, in addition to the *B. juncea* mutants have already provided strong evidence in support of a role for proline in stress tolerance (see next section).

While there is only a small number of true mutants for analysis, a great deal of information has accumulated from studies on lines selected *in vitro*, usually in callus or cell suspension culture and variously described as 'NaCl-adapted' or 'salt-tolerant'. The distinction between the two terms is not always clear. Neither implies a genetic basis for the ability of the cells to grow on saline medium, but the former term perhaps more actively distances itself from the notion. Most reports include evidence for some stability of the trait, *in vitro*, through periods of culture in the absence of stress.

The numerous reports from the laboratory of Hasegawa, on salt-adapted tobacco cell lines, include one of the first to relate salt adaptation to the production of a 26 kDa protein (Singh *et al.*, 1985), which was subsequently shown to be localized in inclusion bodies in the vacuole (Singh *et al.*, 1987). The same group has also demonstrated, by X-ray microanalysis, vacuolar localization of NaCl (Binzel *et al.*, 1988), an observation consistent with the role of NaCl in osmotic adjustment, but at variance with earlier observations (Dix *et al.*, 1986), in which similar levels of $Na^+$ ions were found in the vacuole and cytoplasm of salt-tolerant *Nicotiana sylvestris* cells, with high plastid concentrations in some cells.

Salt-adapted tobacco cell lines have also been used to examine membrane transport processes associated with the use of NaCl for vacuolar osmotic adjustment. Content of a 69 kDa $H^+$-ATPase in tonoplast membrane fractions is reduced in response to salt adaptation, yet its activity exhibits a fourfold increase in $H^+$ transport capacity, and a threefold increase in ATP hydrolysis. This quantitative and qualitative alteration in the primary tonoplast $H^+$ pump is thought to be linked to the vacuolar accumulation of $Na^+$ and $Cl^-$ ions (Reuveni *et al.*, 1990). An increase in $K^+$ uptake has also been observed in salt-adapted cells (Watad *et al.*, 1991). This is presumably to ensure maintenance of adequate $K^+$ levels in the face of competition with other cations, and may result from a combination of increased activity of the plasmalemma $H^+$-ATPase, as already observed for the tonoplast, and a greater selectivity of $K^+$ uptake processes. A correlation between salt adaptation and a prevalance of hexaploid cells has also been found in these cultures (Kononowicz *et al.*, 1990). The authors however suggest that hexaploidy is not the basis of salt tolerance, but rather that high salinity induces endopolyploidy, while selecting against ploidy levels greater than hexaploid.

Salt-tolerant cells lines of alfalfa (*Medicago sativa*), are also proving valuable for molecular studies on salt tolerance. Winicov *et al.* (1989) observe both constitutive and NaCl-inducible alterations in the *in vitro* translation products from tolerant, compared with sensitive cell lines.

Furthermore, they find that most of the changes in mRNA composition are different from those induced by salt in the sensitive line, suggesting the mechanism for tolerance is different from the normal adaptive processes. One 27 kDa translation product found in both tolerant and sensitive (NaCl-induced) lines, may equate to the previously reported (Singh *et al.*, 1985) 26 kDa protein termed 'osmotin'. Winicov and Button (1991) also observe preferential accumulation of a number of photosynthesis-related, plastome-encoded, transcripts in the salt-tolerant lines of alfalfa. Other recent comparative studies on salt-tolerant and sensitive cell lines are mainly restricted to ion uptake properties. For example, Muralitharan *et al.* (1990) observe increased levels of $Na^+$, $Cl^-$ and $K^+$ in selected lines of blueberry (*Vaccinum corymbosum*), using flame photometry and electron microprobe analysis, and suggest NaCl accumulation as the mechanism for improved growth at moderate salinities.

### The role of proline in stress tolerance

The possibility that proline has a significant adaptive role in relation to environmental stresses has long been known (Greenway and Munns, 1980), and it has been shown to accumulate in both salt-tolerant and salt-sensitive cell lines (Dix and Pearce, 1981; Watad *et al.*, 1983). A number of studies on mutant cell lines resistant to proline analogues, and accumulating proline, have indicated improved tolerance to salinity (Dix *et al.*, 1984; Ricardi *et al.*, 1983; Van Swaaij *et al.*, 1986). In hydroxyproline resistant potato cell cultures proline accumulation has also been invoked in improved frost tolerance (Van Swaaij *et al.*, 1986). It is satisfying therefore to find the role of proline being reinforced in recent well-characterized stress-tolerant mutants. *N. plumbaginifolia* mutants selected as resistant to NaCl, KCl and physiological drought (polyethylene gycol) all show constitutively elevated (10–15-fold) levels of free proline, which are further induced to much more dramatic extents than the sensitive control by the appropriate stress (Sumaryati *et al.*, 1992). The results suggest a mutation simultaneously enhancing proline production by deregulation of its synthesis, and increasing its stress-inducibility. Particularly telling is the support for these observations from studies on the proline status of sets of mutants of *B. juncea*, isolated and characterized in two independent laboratories (Jain *et al.*, 1991; Kirti *et al.*, 1991).

### Low temperature stress

As in the case of salinity tolerance, there is considerable evidence for a potential for *in vitro* selection as a means for improving frost tolerance, or the capacity for cold acclimation. Genetic analysis of genotypes differing in cold tolerance, most recently in chickpea (Malhotra and Singh, 1991), suggest dominance of the trait and both additive and non-additive gene effects, while somaclonal variation had led to improved frost tolerance in wheat (Lazar *et al.*, 1988). It is also for this species that the first report of heritable selection of frost tolerance selected *in vitro* has been published (Kendall *et al.*, 1990). These authors bypassed the problem of devising a selection strategy based on the possible need for cold acclimation treatment, and freezing conditions normally causing damage, by using the extreme approach of cryoselection, i.e. subjecting embryogenic calli to deep freezing and transfer to liquid nitrogen, without the use of cryoprotectants. R2 progeny of selected lines showed greater tolerance to moderate (−12°C) freezing conditions than controls. Characterization of one frost-tolerant callus line indicated that neither fatty acid composition nor tissue freezing point were significantly altered, although there was an increase in soluble sugar. Differences in protein components of microsomal membrane fractions were also detected, with the loss of nine polypeptides, and the appearance of seven novel ones in the cryoselected callus. Clearly there is potential for a more thorough investigation of the molecular basis of frost tolerance, preferably carried out on the progeny, in this material. Analysis of other kinds of mutant can also throw light on frost tolerance and cold acclimation. The role of proline in conferring improved frost tolerance on potato cells (Van Swaaij *et al.*, 1986) has already been alluded to. Mutants deficient in or insensitive to ABA, while not selected *in vitro*, can provide valuable leads in the elucidation of the role of this hormone in cold acclimation. For example, Gilmour and Thomasow (1991) have shown that ABA deficient mutants of *Arabidopsis thaliana* do have impaired capacity for cold acclimation compared with the wild-type, and are also more ion-leaky. They were, however, unable to demonstrate any change in the expression of three cold-regulated (cor) genes.

### Heavy metal tolerance

Several heavy metal-tolerant lines have been selected in cell cultures. In contrast to aluminium-tolerant lines, which tend to chelate the ions in the medium, through the secretion of citrate (Ojima and Ohira, 1986), most heavy metal-tolerant lines so far characterized do accumulate the ion in question. They are therefore valuable for fundamental studies on cellular tolerance mechanisms. Cadmium-resistant cell lines of *Datura innoxia* accumulate sulphur-rich metal-binding polypeptides, poly (γ-glutamylcysteinyl) glycines which bind the cellular cadmium (Jackson *et al.*, 1987). These 'phytochelatins' are synthesized from glutathione and it is noteworthy that glutathione-deficient mutants of yeast have greater cadmium sensitivity (Glaeser *et al.*, 1991). Phytochelatins may have a role in

tolerance to other heavy metals comparable with metallothioneins in other organisms, and analysis of mutant plant cell lines should provide further insights into the role of glutathione metabolism in plant stress responses. Kishinami and Widholm (1987), however, invoke chelation by citrate or malate as the mechanism for Cu and Zn tolerance in *N. plumbaginifolia* cell lines.

## Mineral efficiency

In view of the obvious importance of mineral nutrient supply and uptake for crop productivity, it is perhaps surprising that so little attention has focused on the clear attraction of cell cultures, growing in defined medium, to study mineral deficiency. There have, however, been some recent developments in this area. Quantitiative inheritance of tolerance to low-phosphate stress has been demonstrated for several species and has recently been supported by RFLP analysis in maize (Reiter *et al.*, 1991) which indicates six RFLP marker loci associated with performance under low phosphate conditions. A tomato cell line resistant to phosphate starvation has been selected from a cell suspension culture (Goldstein, 1991). Its growth in 1.25 mM Pi medium is the same as the non-selected control, but it performs significantly better at 0.1 mM Pi. The author demonstrates a greatly enhanced rate of phosphate uptake in the selected line, at both levels of Pi, and a constitutively enhanced secretion of acid phosphatase. The implication of these pleiotropic effects is that the phosphate efficient line exhibits an alteration in the coordinated expression of several genes regulating phosphate uptake.

Another mineral nutrient for which deficiency/efficiency has been examined *in vitro* is iron. Stephens *et al.* (1990) used tissue culture to evaluate efficiency of iron utilization in cultivars of soybean, while Naik *et al.* (1990) selected iron-efficient lines from sugarcane callus. Shoot regeneration was accomplished and the resulting shoot cultures were able to grow vigorously on the Fe-stress medium, but further characterization is awaited.

## Conclusion

The analysis of mutants is an important tool for elucidating the basis of tolerance to environmental stresses. The particular attractions of mutant cell lines are twofold. Firstly, *in vitro* techniques provide a handle to obtain mutants difficult to select at the whole plant level and, secondly, characterization of cell lines allows one to assess basic cellular processes, divorced from the organizational complexity of the whole plant. Clearly studies of this kind can only complement whole plant studies, and should contribute to integrated strategies for investigating the molecular and cellular basis of stress tolerance. To date, this contribution has been relatively minor, but some of the studies mentioned here, particularly those on salt- and metal-tolerant lines, clearly indicate a greater role for the generation and characterization of mutant cell lines altered in their response to specific stresses.

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