Recent advances - cell culture in plant improvement

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Introduction

Plant breeding employs a genetic strategy to meet the continual challenge to sustain crop productivity. Diseases and pests, changes in both agronomic practice and consumer demands, climatic perturbations and lessening the need for energy-dependent inputs are components of this challenge. Moreover, to meet the humanitarian imperative of an expanding world population and the economic compulsion of an increased per capita consumption in likely trading partners, Australia, along with other agricultural commodity exporters, will have to sustain an average rate of increase of food production of around 2% per annum.

To meet such peremptory demands the plant breeder will have to employ new and more efficient technology. Several potentially valuable innovations derive from developments in plant cell culture. These include the application of cellular selection to recover useful genetic variants, haploidy to hasten the attainment of homozygosity, embryo culture and somatic hybridization for recombining genomes of sexually incompatible species and greatly increased rates of clonal amplification of asexually propagated species. Further downstream there is a possibility of specific gene addition or modification by recombinant DNA technology. Such possibilities, however, require the development of an efficient tissue culture cycle in the crop species of choice.

A Plant Tissue Cycle

For an increasing number of species, cell lines can be induced to proliferate under defined culture conditions. Plant tissue culture medium consists of defined amounts of inorganic salts, trace elements, vitamins, a carbon source for energy and phytohormones. Virtually any part of a plant can be induced to form a cell line, including the root and stem section, hypocotyl, cotyledons, leaves and even immature haploid pollen grains.

Cultured plant cells have the enormous advantage of totipotency; i.e., rapidly growing undifferentiated cell lines can be induced to form shoot and root primordia which develop into fully fertile plants. The principle determinant of the differentiation process is the relative levels of auxins and cytokinins in the culture media. Generally, a high ratio of cytokinin to auxin induces shoot formation while the converse tends to favour root initiation. For any given species, the culture conditions which favour rapid undifferentiated cell proliferation as against plant regeneration is arrived at empirically.

A tissue culture cycle requires that a cell line be established relatively easily, that the cell line proliferate reasonably rapidly and, finally, that mature fully fertile plants can be regenerated in large numbers at will.

Plant propagators and the nursery industry were quick to see the advantage of tissue culture in terms of rapid propagation of desirable genotypes. There is now a large number of plant species which are amenable to rapid propagation by tissue culture technology (l,2).

In recent times geneticists and plant breeders have successfully applied these techniques to many of our important agriculture crops. Both legume and cereal species, until recently considered recalcitrant to tissue culture, have yielded to persistent efforts. Tissue culture approaches are now being used in lucerne improvement (3,4). Initial progress has been made by (5) and ourselves in developing a tissue culture capability with wheat. The principal objective of these efforts is to augment the armoury of conventional plant improvement. Exciting consequences emanate from these developments.

Somaclonal Variation

It often happens that valuable scientific applications emerge from unexpected quarters. Because a tissue culture cycle was seen essentially as cloning a particular genotype it became the accepted dictum that all plants arising from tissue culture should be exact copies of the parental plant. Phenotypic variants which occurred among regenerated plants were dismissed as "artefacts of tissue culture". The variants were viewed as transitory consequences from exposure to phytohormones and were labelled as epigenetic events which somehow made them unworthy of further scientific interest. Now it seems this was a premature and erroneous judgement. Tissue culture appears to be an unexpected) rich and novel source of genetic variability. This variation is called somaclonal variation and has been reviewed in detail by Larkin and Scowcroft (6).

A. Sugar cane

Initial developments to utilize somaclonal variation in plant improvement began in the Hawaiian Sugar Planter's Association Experiment Station. Variation was observed in sugar cane somaclones for morphological, cytogenetic and isozyme traits (7). Following this, somaclones of several sugar cane varieties grown in Fiji were screened for reaction to Fiji virus disease and Downy Mildew (Sclerospora sacchari). In each case a number of somaclones were identified with increased resistance to either Fiji virus disease or Downy Mildew (8). Similarly, follow up work in Hawaii, where large numbers of somaclones were screened for resistance to Fiji virus disease or eyespot disease (Helminthosporium sacchari) yielded substantial numbers of disease- resistant plants (9).

Sugar cane improvement in Taiwan has also adopted a cell culture approach (10). From as few as 60 somaclones of a smut (Ustilago scitaminea)- susceptible, but otherwise highly desirable cultivar, a line with equal agronomic performance and improved disease resistance has been recovered. Other somaclonally derived variants have shown substantially increased sugar yield under field trial conditions, the best of which show promise for varietal release.

Our own research to explore the potential of somaclonal variation to improve Australian sugarcane cultivars began in 1979. We were concerned with disease resistance and specifically eyespot disease. All lines were derived from the cultivar, Ql01, which is agronomically valuable, except that it is susceptible to eyespot disease. A very efficient cell culture cycle was developed (11) which allowed the continual regeneration of plants from cell lines maintained in culture for up to 30 months.

To facilitate screening a large number of somaclones, a leaf bioassay was developed to quantify the sensitivity of the leaves of a given plant to a standardised concentration of the fungal toxin responsible for leaf damage.

The sensitivity was measured as the initial rate of leakage of electrolytes from leaf discs briefly exposed to the toxin. This metric proved to be highly repeatable and consistent for a given cultivar (12).

Among 350 Q101 somaclones evaluated for toxin sensitivity there was a staggering amount of variability (see 6 and 13 for details). The mode of the distribution of somaclone reaction to the toxin is shifted toward the resistant side of Q101 with about 20% of the somaclones being essentially immune to the toxin. The inclusion of toxin during cell culture further biassed the distribution toward resistance. Many of these toxin-resistant plants are being field tested in Queensland by the Bureau of Sugar Expt Stations.

The stability of 85 toxin-resistant somaclones has been assayed in subsequent vegetative generations. Toxin resistance was retained in 73% of somaclones, 19% had segregating setts and 8% reverted towards sensitivity.

We have also established that a second tissue culture cycle of toxin-resistant somaclones yields secondary somaclones which either retain or have increased resistance, relative to the primary somaclone, in 62% of cases. The remainder have become more susceptible. Thus we can envisage that sequential improvement for several characters is possible using tissue culture. A useful proportion of

those somaclones, initially selected for one character, will retain that trait following a second tissue culture cycle, i.e. characters can be "stacked".

B. Potatoes

Shephard et al. (14) proposed "character stacking" in their development of a tissue culture approach to potato improvement. Having developed a protoplast culture protocol for potatoes, Shephard et al. (14) screened an original population of 1700 protoplast derived plants and found extensive variation for tuber morphology, plant habit, photoperiod response, maturity date and yield. Some of these somaclones (protoclones) were agronomic improvements over the parental cultivar, Russet Burbank.

It is most significant that some somaclones were recovered which were resistant to disease pathogens. Five of 500 somaclones were more resistant to Alternaria solani toxin than the parent and of these, four showed field resistance to early blight. About 2.5% (20 of 800) somaclones screened were resistant to late blight (Phytophthora infestans), some of which were resistant to multiple races of this pathogen. These variant somaclones have retained their phenotype through a number of vegetative generations.

Subsequently, 65 selected somaclones were analysed in detail for culture induced variability under field conditions (15). Among 35 characters analysed, significant variation was found for 22 characters. All clones differed from Russet Burbank for at least 1 character and one somaclone differed for 17 characters.

Protoplast culture technology has now been applied to a number of cultivars in both the USA and England and in those cases where the somaclones have been analysed in detail similar variation to that described by Shephard has been reported (16).

While protoplast culture provides single cell identify for somaclones, it does not appear essential for generating variation. Different somaclones derived from a single protoplast derived callus also showed variation (16). This is strong evidence that variation arose during callus growth rather than reflecting preexisting genetic differences in the cells of the leaf.

C. Cereal Species

Relatively efficient cell culture is now possible in a number of important cereal crops, such as maize, rice and more recently wheat. Already there is substantial evidence that somaclonal variation exists in these seed- propagated species. From callus of a dihaploid rice line (derived from anther culture) Oono (17) found variation among some 800 somaclones screened for chlorophyll content, flowering date, plant height, fertility and plant morphology. Only 28% of plants were considered normal, i.e. parental-like, for all of these characters. Many of these changes had a genetic basis because they were transmitted through a sexual cycle. Initial results from a tissue culture program with rice at the International Rice Research Institute confirms the occurrence of somaclonal variation (Zapata, pers comm).

Somaclonal variation in plants regenerated from maize cell lines, first observed by Green (18), also appears to be extensive. Interestingly this variation can affect the mitochondrial as well as the nuclear genome. Selection for resistance in cell lines of T-cytoplasm maize (which is sensitive to the southern corn leaf blight T-toxin of Drechslera maydis Race T.) by recurrent sublethal exposure to T-toxin resulted in the recovery of toxin-resistant plants. The phenotypic changes were maternally inherited,which correlates with the finding that resistance/sensitivity is a mitochondrial function (19). Brettell et al. (20),in confirming this result, also found that among plants regenerated from non-toxin selected cultures about half of the plants were toxin-resistant and also male-fertile. Endonuclease restriction analyses of mtDNA from toxin-resistant, male-fertile somaclones indicated that the mitochondrial genome had undergone sequence changes relative to the parental T-cytoplasm mtDNA (21). Moreover, the changes that occurred during cell culture were not reversions to the N cytoplasm mtDNA pattern of male-fertile, toxin-resistant genotypes (22). The changes in mtDNA which occur during cell culture have not been observed during conventional seed propagation of maize.

Only with seed-propagated species can genetic credibility be rapidly ascribed to somaclonal variation. Edallo et al. (23) screened the progeny of 77 fertile maize plants regenerated from cultured for a number of simply inherited endosperm and seedling mutations. Only segregations in accordance with Mendelian segregation were considered. The astounding result was that on average each somaclone derived from cell culture carried at least one mutant affecting a major morphological trait. One can only speculate on the number of more subtle mutational events that might have occurred.

D. Other species

Somaclonal variation has now been reported in an extensive array of species. Interestingly many of these species are of economic importance. They include cereals (oats, barley, sorghum), legumes (lucerne, clover), brassicas (cauliflower, rape), vegetable crops (lettuce, garlic, tomatoes), forage grasses (lolium, festuca, panicum) and pineapple. Among horticultural species new cultivars of pelargonium and begonia have been produced from cell culture variants. In the doyen of plant tissue culture, tobacco, substantive independent experiments confirm the reality of somaclonal variation as a real genetic phenomenon.

E. Origin of Somaclonal Variation

An understanding of the causes of somaclonal variation is important, first to be able to increase the level of variation where increased variability is the objective. Second, where the goal is to propagate a cultivar clonally to produce uniform progeny the ability to suppress the phenomena could be very desirable.

Doubtless many different genetic processes operate to generate somaclonal variants. A discussion of some of the possible processes can be found in Larkin and Scowcroft (6). Gross karyotypic changes and chromosomal rearrangements have been documented in both cell cultures and in regenerated plants. However, these gross changes seem to reflect the capacity of cell culture to generate variation rather than as an underlying cause of phenotypic variability. While abnormal karyotypes have been observed in somaclones of potato and pelargonium, for example, these represent only a small proportion of the total phenotypic variants observed.

Cryptic chromosomal rearrangements leading to translocations, deletions and inversions, for example, could have a genetic consequence affecting an individual phenotype. For example, a small rearrangement may delete or otherwise switch off a dominant allele, thereby allowing the recessive allele to affect the phenotype.

There is evidence in higher organisms, including plants, of a phenomenon where particular stretches of DNA (transposable elements) can move from one locus to another at relatively high frequency. The transposition of such elements from one chromosomal location to another generates mutations. A tissue culture environment may be conducive to sequence transposition. This appears to be the case in animal cell cultures. Such a high frequency transposition would generate extensive phenotypic variability.

It has been shown for higher organisms, including plants, that the quantity of specific gene product can be increased or decreased simply by amplification or diminution of the gene copy number. The artificial nature of a tissue culture environment may impose sufficient selection pressure to cause both amplification of some genes and diminution of others. This would obviously affect the phenotype of plants regenerated from the cell lines so affected.

Table 1. Cell culture selection for agronomically useful genotypes

Selected trait	Species	Plant regeneration and expression	Inheritance	References
1. Disease (toxin) tolerance				
Pseudomonas tabaci[†]	Nicotiana tabacum	+	Semi-dominant	Carlson (26)
Helminthosporium maydis	Zea mays	+	Cytoplasmic	Gengenbach et al (19) Brettell et al. (20)
H. sacchari	Saccharum officinarum	+	?[††]	Larkin and Scowc (in prep.)
Phytophthora infestans	Solanum tuberosum	+	?	Behnke (27)
2. Herbicide tolerance				
Picloram	N. tabacum	+	Dominant or semi-dominant	Chaleff and Parsons (28)
3. Physiological tolerance				
NaCl	N. tabacum	+	Nuclear?	Nabors et al.
Sea water	Oryza sativa	+	Nuclear	Yamada (pers
4. Nutritional Quality				
Enhanced threonine	Zea mays	+	Dominant	Hibberd and G (25)

[†]A pseudo-analogue of wildfire toxin, methionine sulfoximine, was used as the selective agent
[††]Stable for at least 5 normal sett generations.

Cell Selection and Genotype Improvement

Not only can cell culture be utilised to generate genetic variation but it also offers the capacity for direct cellular selection of potentially useful genotypes.

Progress in the development and success of cell culture selection systems has been well documented (24). Most success has been achieved in selecting for amino acid analogue resistant cell lines, metabolic deficiencies and drug resistant mutants. Few of these are of direct relevance to agriculture, except perhaps those mutants which result in the overproduction of certain amino- acids.

One recent example where cell selection has led to increased threonine content in maize kernels illustrates the power of this technique (25). A maize cell line was selected for resistance to growth inhibition by equimolar concentrations of lysine plus threonine. Genetic analysis of plants regenerated from the selected line showed that the resistance was inherited as a single dominant nuclear gene. Total threonine in plants homozygous for the selected gene was increased by 33-59%. This could be a significant advance in increasing nutritional quality of maize, for which threonine is the 3rd most limiting essential amino acid.

Of even greater importance is the potential of cell culture to increase disease resistance and physiological and herbicide tolerances. Table 1 lists those cases where selection in cell culture has been employed to produce plant genotypes of relevant economic value. This list is restricted only to those cases where regenerated plants have been tested for their response to the selective agent and,where possible, genetic analyses carried out. There are additional cases where selection has produced cell lines with increased tolerance or resistance but the response of regenerated plants has not yet been reported. These include tolerance to aluminium in tomato (30), dimethylsulfoxide (a possible membrane stress analogue) in wheat (31), and chilling in tobacco and capsicum (32),where regenerated plants neither expressed the trait nor transmitted it to progeny (33). Cell lines tolerant to herbicides such as 2,4-D and 2,4,5-T in Trifolium repens (34),aminotriazole in N. tabacum (35) and 2,4-D in lucerne have been recovered.

Conclusion

The limited though striking successes that have already been achieved through selection in cell culture has encouraged considerable activity in this research. The cell selection approach is of course limited to those whole plant characteristics which are known to have or suspected of having a substantial cellular component. This can often be established by correlating cellular and whole plant response to the agent of selection. Doubtless the reductionism of plant biochemistry and molecular biology will open up new approaches to cellular selection.

Together cell selection and somaclonal variation provide exciting and we hope powerful new technologies for plant improvement. They provide new sources of variation and more efficient means of enriching for particular variant genotypes. They do not, however, provide a panacea. Any benefits this technology can offer can only be achieved by mutual interaction with plant breeders and other plant biologists.

A Postscript - What about wheat?

In the Division of Plant Industry, we have recently begun to develop a tissue culture capability with wheat. Along with others elsewhere (5,36) we have developed a relatively efficient tissue culture protocol for wheat to facilitate the regeneration of large numbers of plants which can be transplanted to the greenhouse with minimal loss. An important component in this development was the use of immature embryos to initiate the cell lines. About 50 cultivars and closely related species of T. aestivum have been screened for cell culture capability, including 20 Australian cultivars. Some of these, such as Millewa, Lance, Warigal, Gamenya, Egret, Bindawarra and the old stalwart, Bencubbin, perform extremely well. Our initial observations on plants regenerated from cell culture indicate that somaclonal variation is alive and well in Triticum. We intend to evaluate somaclonal variation at loci controlling reaction to rust pathogens, several morphological and biochemical characters, and to attempt cell selection for herbicide tolerance and Septoria disease resistance.

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