CELL CULTURE MANIPULATIONS AS A POTENTIAL BREEDING TOOL

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I. INTRODUCTION

Protagonists of plant tissue and cell culture methods are now commonplace. Applications of these techniques can be found in nearly every field of plant science and they have resulted in many valuable contributions to our knowledge of primary and secondary metabolism, the cell cycle, the regulation of growth and differentiation, and plant/micro-organism interactions. From the agricultural point of view the obvious attractions of tissue culture methods for virus eradication and rapid clonal propagation of commercial varieties have continued to draw a great deal of attention and the number of species for which the basic culture criteria have either been met, or are under intensive study, is now quite extensive (43). Interest in genetic manipulations of plant cell cultures has continued to increase, despite the many obstacles in the way of wide application of these methods. It is therefore expected that those interested in overcoming low temperature stress would also direct some attention to these genetic manipulations.

This review will consider the different methods for genome modification in cell cultures and the problems encountered in the application of these methods. The extent to which these problems have been and can be overcome will be evaluated with particular reference to experiments performed with crop species, and having a potential for crop improvement. In addition the limited progress which has been made in breeding for chilling resistance using tissue cultures will be considered.

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II. THE CURRENT STATE OF PLANT CELL CULTURE TECHNOLOGY

The basic cycle of initiation of undifferentiated cultures, and regeneration of intact plants from them has now been demonstrated for a large number of species and in many of them finely dispersed cell suspension cultures, and protoplast isolation, culture and fusion, have extended the range of methods which can be used to effect genetic modification. In addition anther culture, first used by Guha and Maheshwari (27) has resulted in the availability of haploid material in a far greater range of species than before (50).

It remains true, however, that for a large number of valuable crop species all the essential culture conditions have not been worked out, and for many others they have yet to become sufficiently refined and repeatable as to provide a really useful breeding tool. Certain families, most notably *Solanaceae,* have proved generally more amenable to cell culture manipulations than others. Tree species and cereals have tended to prove recalcitrant although the concentration of effort and resources has begun to yield a measure of success in several cereal species (43). Most commonly the regeneration of plants from cell cultures is the critical stage.

III. THE USE OF CELL CULTURES IN BREEDING

A. *Exploitation of Existing Variation*

Anther-derived haploid plants and homozygous diploids derived from them by colchicine treatment (28) provide a means of accelerating the exploitation of existing variation by conventional combination breeding (39). This procedure has already been successfully used to develop new cultivars of tobacco (45, 9) and is certain to be extended to other crop species as the anther culture procedure becomes more widely applicable and reliable.

B. *Mutation and Selection*

1. Selection Procedure. Selection in cell cultures is only useful for characters which are likely to be expressed equally well in undifferentiated cells and intact plants. Selection procedures usually involve the exposure of callus, cell suspension, or protoplast, cultures, with or without a prior mutagenesis step, to a suitable selective treatment which kills or inhibits the division of normal cells. Surviving cells give rise to healthy proliferating aggregates which can then be repeatedly exposed to a cycle of selection and regrowth, and plants regenerated from them.

2. Problems

a. Mutagenesis. The number of cells present in a culture make it realistic to select mutants without recourse to a mutagenic treatment, and this was the case with many of the variant lines described here. Effective use of mutagens has been described in some cases (51, 42). It may be wise to restrict the use of mutagens as far as possible when attempting to modify crop plants.

b. Aggregation. Except in the case of protoplasts, cell cultures do not exist predominantly of single cells, but of aggregates of various sizes, and cannot therefore be regarded as microorganisms. Within an aggregate cells may be of different sizes and physiological states, and intercellular connections, together with a variety of gradients across aggregates could interfere with the selection of some kinds of variant.

c. Minimal cell density. Most cell cultures require a minimum cell density for growth which may pose problems when trying to select a few survivors from a large population of dead cells.

d. Chromosomal instability. A well known feature of cell cultures is the chromosomal instability induced by the culture system which over a period gradually gives rise to polyploid or aneuploid cells (48, 11, 1). This does not occur in all species but appears to be particularly pronounced in haploid cultures (48). Gross chromosomal changes obviously do not favour the use of cultures in breeding.

e. Loss of morphogenic potential. The potential for the initiation of shoots or embryos in culture is often reduced by an extended culture period, a phenomenon possibly associated with *d*. This is the most commonly encountered problem in variant selection in cell cultures. The majority of variant cell lines which have been described exist only as cell lines. These two features together mean that expedient use of freshly initiated cultures is likely to remain desirable when crop improvement is the aim.

f. Loss of flowering or fertility. May be further consequences of culture induced incidental genetic changes. Loss of flowering has been found in plants regenerated from a streptomycin resistant cell line of *Nicotiana sylvestris* (37).

g. Epigenetic variation. Phenotypic changes, resulting from causes other than mutation (such as changes in gene expression) can often be found in cell cultures. An example is cycloheximide resistance (35). Some epigenetic variants may be very persistent.

Many of these problems can be avoided, or may not exist for a particular system, and, in spite of them a number of variant lines have been selected in culture. In some of them (38, 33 & 34, 3), sexual transmission has been unequivocally demonstrated. There follows a brief survey of the variant lines which have been selected for characters of potential agricultural interest.

3. *Disease Resistance.* Carlson (5) produced tobacco plants resistant to infection by *Pseudomonas tabaci* by selection for resistance to methionine sulfoximine, an analogue of the toxin, in haploid cell cultures. Gengenbach and Green (22) selected for resistance to the toxin of *Helminthosporium maydis* in Texas male-sterile maize cultures to obtain plants resistant to the pathogen. Certainly the most significant case is the development of sugar cane cultivars resistant to four different pathogens (46).

4. *Herbicide Resistance.* Chaleff and Parsons (6) have demonstrated the sexual transmission, as a dominant allele, of picloram resistance selected in tobacco cultures. Several other cell lines resistant to herbicides have recently been selected (26).

5. *Environmental Stress Resistance*

a. Low temperature. Dix and Street (13) obtained cell lines of *Nicotiana sylvestris* and *Capsicum annuum* with enhanced resistance to exposure to $-3^{\circ}C$ and $5^{\circ}C$ respectively. Two types of resistant line of *N. sylvestris* differed markedly in their level of resistance. Unfortunately plants could only be regenerated from some of the lines with a lower level of resistance and callus derived from the seedling progeny was sensitive (10). Plants could not be regenerated from resistant or sensitive *C. annuum* cultures. It is concluded that the lower level of chilling resistance in *N. sylvestris* resulted from a fairly stable epigenetic change, persistent through an indefinite number of mitotic divisions in cell culture, but lost during the plants sexual cycle. Alternative explanations for the loss of resistance, such as continual segregation in culture giving rise to chimeral plants cannot be ruled out.

The selection system used in the above work resulted in many lines which survived the first selection, but succumbed to subsequent exposure. This may reflect the physiological spectrum of cells present in the culture simultaneously, and the problems of variations in aggregate size already discussed. These factors may have a greater impact on the selection of this kind of variant than, for example, on selection for resistance to most drugs. This may be the more so since we are looking for survival and growth subsequent to the selection pressure, rather than growth in the presence of a selective agent. Considerable refinement of the selection procedure and a thorough examination of a large number of putative mutants should eventually lead to cultivars with enhanced chilling resistance. It is encouraging that the possibilities are being further investigated using cell cultures of tomato (4) and rice (Xuan and Dix, unpublished).

b. High salinity. The selection of cell lines with enhanced resistance to growth inhibition by sodium chloride has been described for *Nicotiana sylvestris* (56, 12), *Nicotiana tabacum* (44), *Capsicum annuum* (12), and *Citrus sinensis* (30). In the case of *N. sylvestris* a number of plants have been regenerated from a resistant cell line, and calli initiated from them retain their resistance (Dix, unpublished).

c. Aluminium. Meredith (41) has reported the selection of cell lines of *Lycopersicon esculentum* with a stable resistance to aluminium toxicity.

6. Amino Acid Overproduction. A common mechanism for resistance to amino acid analogues is a reduced sensitivity of the feedback control mechanism of the biosynthetic pathway for the appropriate amino acid, resulting in its overproduction. If this could be translated into improved levels of key amino acids in storage organs or seed proteins the potential for crop improvement would be enormous. For this reason more attention has been paid to selection for amino acid analogue resistance in cell cultures than to any other class of mutant, and several recent reviews have covered the subject (54, 55, 32). Many of the analogue resistant lines which have been selected are indeed overproducers of the corresponding amino acids but in none of these has overproduction been shown in regenerated plants, or sexual transmission been demonstrated. S-2-aminoethyl-cysteine (AEC) resistance selected in barley embryos (Bright, personal communication) and methionine sulfoximine resistance selected in tobacco cultures (5) were sexually transmitted as recessive traits, but the mechanism of resistance was not known in these cases.

The potential of cell cultures for the selection of this kind of mutant has been well illustrated by Widholm (53). Using sequential selection for resistance to four different analogues, he has obtained a carrot cell line simultaneously overproducing lysine, phenylalanine, methionine and tryptophan.

C. *Somatic Hybridization*

1. Protoplast Isolation, Culture and Fusion. The main attraction of somatic hybridization lies in the possibility of surmounting the compatibility barrier between species, and the creation of novel hybrids, but protoplasts may also prove the most realistic vector for the transfer of nuclear and cytoplasmic genes from one species to another. The range of species for which the isolation and culture methods have been met, has been covered by several recent reviews (21, 52, 19, 20). Protoplasts can now often be obtained from callus and cell suspension cultures, as well as a wide range of plant organs. Fairly general methods for improving the frequency of fusion between protoplasts have been developed, probably the most popular being the use of polyethylene glycol (PEG) in a method devised by Kao and Michayluk (29).

2. Hybrid Selection. The products of protoplast fusion are heterokaryons which may then go on to form hybrids by nuclear fusion. It is then necessary to select out hybrid cells from the large majority of unfused cells, and self-fused cells. Most procedures, well reviewed (7), involve complementation resulting in preferential growth of the hybrids under certain selective conditions. For this the parent species or cell lines must be carefully chosen and mutant cell lines can be particularly useful. Complementation between two non-allelic mutant cell lines has been used, as in nitrate reductase deficient mutants of tobacco (25), but a single mutant line can also be combined with a visually distinct one. For example, Maliga*et al.* (36) fused protoplasts of an albino kanamycin resistant line of *Nicotiana sylvestris* (14) with *N. knightiana* mesophyll protoplasts which divide at a low frequency to give green colonies. Hybrids were selected as green kanamycin resistant colonies.

Where mesophyll protoplasts are fused to those of a cultured cell line, which is normally unpigmented, heterokaryons can often be visually identified and physically separated using a micropipette (40). This may prove a more general procedure for the isolation of hybrids from a mixed population. Confirmation of hybridity is generally sought either by comparison with sexual hybrids (when sexually compatible species have been used), by karyotyping, or by looking for intermediate morphological (17) or biochemical characteristics, such as isoenzyme patterns (36).

3. Somatic Hybrids

a. Intrageneric. Somatic hybridization within a species or between closely related species could be a very useful breeding tool. Hybridization in species which take a long time to flower could be accelerated, since seedling protoplasts could be used, and there may be incompatibility factors between quite closely related species. In the event of valuable mutant cell lines being obtained, which have lost their capacity for shoot regeneration or flowering, intraspecific, or intrageneric protoplast fusion may provide a means of utilizing the desired phenotype. It may be possible to regenerate fertile plants from the hybrid cells and eliminate chromosome anomalies in subsequent sexual cycles.

b. Intergeneric. All indications are that the possibility of producing novel hybrid plants between distantly related species remains remote. The biological barrier involved goes far beyond the physical barrier of the cell wall. There is, however, no special problem in producing heterokaryons and hybrid cells, and the range of viable cell hybrids produced in this way has been recently reviewed by Constabel (8) who also considers the fate of the two sets of chromosomes in the hybrids. Generally hybrid formation is followed, during subsequent divisions, by chromosome elimination, a long familiar feature of animal cell hybrids. In some combinations, such as *Vicia* and *Petunia* (2) and *Arabidopsis* and *Brassica* (24), chromosome elimination seems to be non-specific, chromosomes of either or both parents being lost. In other

cases, however, there appears to be specific elimination, of *Petunia* chromosomes from *Parthenocissus* and *Petunia* cells (47) and *Aegopodium* chromosomes from *Daucus* and *Aegopodium* cells (18 and personal communication). This last is of particular interest since plants could be regenerated and although only *Daucus* chromosomes could be detected, certain features of the pigment spectrum of *Aegopodium* were demonstrated. The implication, incorporation of *Aegopodium* genes to *Daucus* chromosomes, could clearly be of greater significance for breeding.

D. Other Methods of Genetic Modification

DNA has been introduced into plant cells and protoplasts in a variety of ways, such as using naked DNA (31), bacteriophage (15), bacteria (23), organelles (23), and animal cells (16). Stable incorporation and expression of exogenous DNA introduced by any of these methods has not been unequivocally demonstrated, and it is difficult to envisage any application to plant breeding in the foreseeable future. More promising is the possibility of transformation using a well characterized plasmid, such as the Ti plasmid of *Agrobacterium tumefaciens*, and this system has been fully reviewed (49).

IV. CONCLUDING REMARKS

To date the increasing use of plant tissue and cell culture methods to achieve agricultural objectives has not been widely extended to the production of new cultivars after genetic modification of cells in culture. Increased experience in tackling the technical difficulties has enhanced the possibility that valuable contributions can be made in this way. It is the view of this author that there are two approaches most likely to be developed as valuable breeding tools used separately or in combination: -

the direct selection for desirable phenotypes in freshly initiated cultures, followed by plant regeneration, and

transfer of parts of genomes responsible for desirable traits using intra- or inter-generic protoplast fusion (chromosome elimination may prove an advantage, rather than a problem, for the application of this method).

With particular reference to the area of interest to this meeting, I know of no serious barrier to the use of these techniques to obtain cultivars of crop species with enhanced resistance to low temperature stress.

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