Plant Cell Cultures: Genetic Aspects of Crop Improvement

The usefulness of plant cell culture techniques will depend on advances in the other plant sciences.

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Even the most optimistic agricultural experts foresee the need of quantum increases in agricultural production (*1*). Central among the obstacles that impede this goal of increased production is the real ceiling imposed by dwindling genetic variation in many crop species (*2*). Recent advances in plant cell and protoplast culture have drawn considerable attention because they form the basis of a novel technology to induce and recover agronomically desirable mutations, to make possible the rapid screening of naturally occurring variability, and to extend the range of plant hybridization beyond the bounds of sexual compatibility (*3*, *4*). In this article, we shall attempt to evaluate current progress in this field and assess the potential for genetic applications of plant cell and protoplast culture both as techniques to expand the range of genetic variability and as tools for plant breeding.

The basic manipulations in vitro that are necessary for the genetic modification of plant cells are just now being developed, so that it is premature to judge the form of the final relationship between cell culture and crop improvement. As the disciplines of culture in vitro and plant breeding are juxtaposed in future research, new techniques will be developed that will permit greater utilization of cellular methods by the breeder. This article attempts to view the current relationships between these two fields. We expect that the results and developments of future years will provide a more complete perspective. At present, the promise of plant cell culture technology appears to lie in two broad areas that are related to our extending the range of genetic variability—that is, parasexual hybridization and mutant selection.

Cell culture techniques offer a vehicle for the application of the methods of molecular genetics to higher plants in those few systems where the necessary manipulations in vitro have been defined (for example, tobacco, *Nicotiana tabacum*, and carrot, *Daucus carota*) (*5*). By employing single haploid or diploid cells as experimental organisms, it is possible to utilize procedures developed with microbial organisms to analyze and modify higher plants. It is now possible to grow large, relatively homogeneous populations of higher plant cells in a short period. This is accomplished, after inducing the cells to divide in a rapid unorganized fashion, by manipulating hormone levels in the culture medium. The resulting proliferation, termed a callus, can be dispersed and grown in liquid medium to generate a suspension culture consisting of single cells or small clumps of cells. Cells can be grown in chemically defined media, and defined mutants can be recovered by imposing chemical selection screens.

By enzymatically removing the walls from plant cells one can obtain large populations of protoplasts. These protoplasts can be induced to fuse with one another, and by this technique a number of interspecific and intergeneric heterokaryons and synkaryons have been produced. In some systems it is possible to stimulate the regeneration of single cells and of protoplasts into entire plants. This is normally accomplished by manipulating hormone levels in the culture medium.

In general, plantlets develop from undifferentiated cells along one of two pathways. With some species, cells in suspension culture can be stimulated to undergo organization directly into a plantlet through stages which resemble normal embryogenesis. With other species, cells from suspension cultures are allowed to proliferate prior to the application of the hormonal stimuli for shoot and root regeneration; hence, organ differentiation occurs within a large cell mass. Since whole plants can be recovered from cultured cells, a standard Mendelian analysis can be performed upon variants recovered in vitro. Because of their properties, plant cells can be manipulated as microbes; the traits they acquire in culture can also be analyzed genetically and physiologically in the whole plant.

Components of Cellular Technology

The specific manipulations in vitro that are of importance to the plant breeder can be summarized as follows.

Cellular cloning and the rapid propagation of genotypes. In numerous species of higher plants, large, genetically uniform populations of individuals can be produced from daughter cells ultimately derived from a single cell (*6*). Such cloning, which could prove important for increasing a superior genotype, can be accomplished with only a minimum of technology. Rapid cellular proliferation followed by plant regeneration from this tissue are the two required manipulations for inducing cloning in vitro. These two manipulations are generally routine in a wide variety of crop species, and have been accomplished even when regeneration from long-term undifferentiated cell cultures has proved impossible. Cellular cloning is currently being utilized in several crop species (*7*).

Induction of haploid tissue from anther and pollen culture. Haploid plantlets and callus tissue can be obtained in a number of genera by the techniques of anther and pollen culture (*8*). Within the immature pollen of cultured anthers, the vegetative cell can undergo division to generate an embryoid or an undifferentiated callus mass with the gametic number of chromosomes. Haploid cell lines are of importance because they permit direct selection for mutant phenotypes, and because they can readily be induced to form diploid tissue that yields isogenic lines. Anther-derived haploid plantlets are currently being used in

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only a few breeding programs (*9*) because they have not been routinely and widely produced among agronomically important crops (*8*).

Extending the range of genetic variability by way of induced mutations. Defined mutant types can be generated and recovered from cells of higher plants cultured in vitro (*3*). Cell cultures offer a microbial-like genetic system because it is possible to examine large numbers of individuals under known conditions, and it is often possible to work with haploid cell lines. Furthermore, individual plant cells have limited metabolic reserves, and their ability to grow on a completely defined, simple media allows selection for a wide range of variants. A number of mutant selection techniques, taken directly from microbial work, have been successfully utilized with plant cells. The direct application of microbial mutant selective procedures to higher plant cells may not be satisfactory for the plant breeder's needs. Microbial mutants have been recovered in order to analyze the biochemical and genetic organization of these cells. The aim of the plant breeder is not so much to analyze processes as to utilize them in maximizing yields. Novel selective schemes will be needed to accomplish this objective.

Extending the range of genetic variability by way of somatic hybridization and protoplast manipulation. A hybridization breeding program in vitro offers the possibility of producing hybrid plants containing genetic information from sexually incompatible species. Hybrid individuals can be produced by utilizing methods that do not involve standard sexual mechanisms (*10*). The isolation of viable protoplasts that undergo proliferation by cell division has now been reported in a number of plant genera (*11*). Furthermore, the fusion of protoplasts from different plant taxa can routinely be induced to occur with a high frequency (*12*). However, the rapid selection of hybrid clones in vitro awaits the development of adequate selective markers for the recognition of hybrid cells. Regeneration of hybrid cells into entire plants also presents a major problem. Protoplasts can be stimulated to take up macromolecules, viruses, organelles, and whole bacteria (*3, 4*). This property, when more completely characterized, may permit the introduction of large blocks of genetic information.

Transfer of specific genetic information. The transformation of genetic information is a common means of genetically modifying bacteria. If this process could be routinely accomplished in higher plants, it might well provide an important tool for the breeder. Although there are claims for the occurrence of transformation-like events in higher plants (*13*), the literature is not clear and no reproducible systems are known that would enable one to examine this phenomenon.

Current technical limitations. At present, the techniques we have described can be utilized with only a few species of higher plants, and in no species are these techniques routine procedures. The most obvious limitation to the manipulation of plant cells in vitro is our inability to achieve the regeneration of whole plants from cell cultures of cultivars of the important crop species. While there is no reason to suspect that this problem is more than technical, genetic variability induced in vitro cannot be utilized by the breeder unless whole plants (and gametes) can be recovered. An equally severe limitation of cellular technology stems from our limited knowledge of plant physiological and biochemical processes. The recovery of plant cells with genetic variation in vitro is dependent upon our being able to recognize distinct cellular phenomena. By further research we should be able to gain some insight into the molecular and cellular mechanisms underlying agronomically important traits. The physiological components of these whole-plant characters must then be duplicated in vitro. Selection schemes for recovering variants in processes unique to higher plants must also be developed. These are only several of the problems that limit the applicability of cell culture techniques to plant breeding. They serve to illustrate the need for further research that will enable us to more adequately define the role that cellular techniques will play in plant breeding practices.

Do These Techniques Offer Unique Solutions to Agronomic Problems?

There are a number of superficial situations in which an attempt to duplicate a natural stress in vitro might result in a cell culture yielding an agronomically interesting variant. Populations of mutagenized cells or of hybrid cells could be exposed to the agent of stress, and survivors could be recovered as potentially interesting individuals. Relatively straightforward selective procedures might be designed to recover, for example, cells tolerant to toxic amounts of specific ions, including hydrogen ions; to pollutants or herbicides; or to extremes of temperature or water stress; or to recover cells that can better utilize available nutrients. Similar schemes might be developed for recovering variants that are resistant to certain diseases or insensitive to pathogens, or that display an improved nutritional quality. It is not yet clear whether such variants would be expressed as positive agronomic changes in the whole plants.

We describe below two examples of situations in which cell cultures might be particularly useful for plant breeders. We think that many other examples of the applicability of cell culture techniques to a wide range of agronomic problems could be found. They represent a different perspective and approach from that normally taken by a plant breeder.

Nutritional Improvement

The protein quality of plant seeds is not of optimal nutritive value for humans. A high proportion of seed protein has been characterized as storage protein and is devoid of any known enzymatic activity. In general, cereal grains are nutritionally poor in lysine, threonine, or tryptophan (*14*). Soybeans and the other legumes are nutritionally limiting in methionine (*14*).

Plant breeders following traditional methodologies are actively engaged in selecting grain and legume varieties with more nutritionally balanced seed protein. The *opaque-2* and *floury-2* mutants of maize, first named for their altered macroscopic endosperm properties, were found to contain greatly enhanced amounts of protein-bound lysine (*15*) as a result of alterations in the relative amounts of lysine-poor and lysine-rich endosperm protein (*16*). Several workers have examined seeds from 9000 lines of sorghum for *floury* endosperm. Of the 62 lines selected, two exhibited, like *opaque-2* corn, a 60 percent increase in seed lysine content (*17*). A barley line, *Hiproly*, with a 20 to 30 percent increase in lysine content, was selected from 1000 entries in the world barley collection by a specific lysine-binding technique (*18*). Other barley lines with a mu-

tagen-induced high lysine content have been isolated but, unlike the *Hiproly* line, these are usually associated with low crop yields (*19*). The points to be made here are that nutritionally beneficial changes in the quality of seed protein have been found through laborious examination of many lines, that selections based on endosperm morphology or dye-binding (*19*) may not select for all beneficial mutants or varieties, and that the numbers of organisms examined are three or four orders of magnitude less than those examined in microbial mutant selection.

Increasing the production of specific amino acids is commonplace in microbiology and is generally accomplished by selecting for mutants resistant to analogs of the given amino acid. This approach has been extended to plant cells, and mutants that display increased endogenous levels of free tryptophan, lysine, and methionine have been recovered (*20*).

There are several problems with this approach, the first one being that one cannot assume that cells that are resistant to the amino acid analog can be regenerated into whole plants. Another problem is that it cannot be assumed a priori that increased amino acid levels will occur in the edible portions (grain, fruit, tuber, leaf) of the regenerated plant. In any case, increasing free amino acids is probably less desirable than increasing tissue-specific levels of the protein-bound amino acid. Free amino acids are more easily leached out in food preparation and represent only a small fraction of the total amino acids in a cell.

A possible approach to effect increases in specific protein-bound amino acids in grains is to elicit production of specific seed proteins in the culture in vitro. If differentiated states of the plant were better understood and could be manipulated, a perpetual differentiated endosperm could be established in culture. At our present level of expertise, however, it may still be possible to select regulatory mutants in the overproduction of a nutritious, naturally occurring seed protein.

Recent work has focused on ways to elevate levels of urease in soybean tissue culture (*21*). This enzyme is particularly rich in methionine, the nutritionally limiting amino acid in soybeans. Although it is a naturally occurring seed protein in a range of leguminous species, the levels of urease in soybean seeds are only 7 to 13 percent of those found in jack bean. Several general selective systems to recover variants have been established in vitro: (i) the utilization of urea in the presence of specific urease inhibitors; (ii) the utilization of urea in the presence of nonmetabolizable repressors of urease production; and (iii) the conversion of large amounts of urea to ammonia to overcome nitrate poisoning in cell cultures. Although no mutants have been positively identified, there appears to be much leeway in elevating the urease levels. Soybean tissue grows quite well with urea as the sole nitrogen source but under such conditions exhibits urease levels only 0.2 percent of those found in seed extracts.

The production of other nonenzymatic seed proteins, for example, zein, gluten, and glutelin, might be manipulated and analyzed in cultured cells if methods were designed for selecting and assaying the variants.

Net Photosynthetic Efficiency

Could a cell line from a crop plant be established in vitro as an efficient *Chlamydomonas*-like photoautotroph? If so, it would be suited for dissecting and genetically modifying the molecular events of photosynthesis and respiration. Modification of these two processes might be important for improving intrinsic plant productivity (*22*). Zelitch (*22a*) has reviewed several reported photoautotrophic cell culture systems. In none of these has the growth rate reached the maximum of cultures supplied with an exogenous carbon source. Selection for photosynthetically efficient cell lines in vitro could be masked by many trivial mutants. Such mutants could be tissue-culture-specific variants, including those with more disperse cell growth allowing better carbon dioxide diffusion, or those with altered optimum hormone requirements for autotrophic growth. Existing photosynthesizing cell lines will have to be altered genetically so that they achieve a higher baseline efficiency before they can be useful in obtaining mutants specific in the carbon-fixing and electron transport processes of photosynthesis.

Net photosynthesis is likely to increase when respiration (essentially the "unfixing" of CO_2) is made more efficient. Photorespiration, which occurs when photosynthetic tissues are illuminated, results from the oxidation of photosynthetic products; it is often more rapid than the rate of respiration in darkness. Evidence suggests that such species as corn and sugarcane are photosynthetically more efficient than other crop plants mainly because they have lower rates of photorespiration; in maize, the primary substrate of photorespiration, glycolic acid, is synthesized more slowly than in species with a rapid photorespiration (*22*). Inhibition of the synthesis or oxidation of glycolic acid results in increased CO_2 fixation in tobacco leaf disks. Because tobacco has a high rate of photorespiration and is amenable to tissue culture, it would appear ideally suited as a system for genetically modifying net photosynthesis through lowering its rates of photorespiration. A potential selection scheme might be one in which recovered cell lines could utilize glycolate as a carbon source. Such lines might exhibit lowered glycolate oxidase levels, or they might acquire the ability to convert glycolate to other metabolites (malate or glycine, for example). Tobacco cell cultures do not utilize glycolate, even under a variety of conditions. Carboxylic acids in general appear to be poor carbon sources for cultured tobacco cells. As with the selection of photosynthetically efficient mutants, the selection of glycolate-utilizing cell lines will probably not initially yield variants specifically altered in photorespiration, but rather in such functions as glycolate uptake. However, once the genetic and physiological groundwork is more completely defined, the genetic manipulation of glycolate metabolism can be expected.

Some of the respiration that occurs in darkness may also be wasteful. An alternate cyanide- (or antimycin A) insensitive oxidase pathway has been described in the fungi and in plant mitochondria (*23*). The phosphate to oxygen ratio of this pathway is probably only one, as opposed to three for the main terminal oxidases. The total respiration of spinach leaves is inhibited only 50 percent by cyanids. Likewise, the alternate oxidase pathway can account for more than 50 percent of the total respiration in tobacco suspension cultures. The alternate oxidase pathway can sustain cell viability for at least 24 hours in suspension cultures when the main cytochrome chain is blocked by antimycin A (*21*). Although a posi-

tive selection method for the elimination of the alternate oxidase pathway has been reported for the fungus *Ustilago maydis*, only a negative selection procedure has been devised to select for this mutation in higher plant cells (*21*). It is difficult to imagine how the plant breeder could select for a specific deficiency in the alternate oxidase pathway at the whole-plant level. Nevertheless, this problem illustrates how plant cell genetics may become increasingly applicable to crop production as the mechanisms of plant biochemistry and physiology are more fully characterized.

Once they have been more fully developed, approaches similar to these should enable plant breeders to obtain useful genetic variants that might be incorporated into crop varieties.

The Future of the Technology

The contributions of cell culture techniques to crop improvement can best be considered as part of an interdisciplinary effort incorporating the approaches of cellular and whole-plant biology. Because cell culture is only one of the many technologies that can be employed in a crop improvement program, the results it yields are dependent upon input, analysis, and evaluation from a number of other disciplines. In a real sense, the usefulness of cellular techniques is dependent upon advances in many areas of the plant sciences.

The history of plant breeding contains instances where new techniques have fostered hopes of an easy method to produce the "ideal" plant type. These techniques have become, with time, the standard tools of plant breeders. We believe that cell culture techniques will also become routine tools in the difficult task of plant improvement. The true test of any developing agricultural technology is how its application affects plant characters under actual field conditions. Viewed from this perspective, cell culture techniques have not yet had the chance to prove themselves. They have not yet produced any novel genetic variants or combinations of genetic characters of agricultural importance.

In spite of the advances that have been made in the use of cell cultures, it does not appear likely that these techniques will be of major importance in increasing crop yields over the next decade. Most of the progress during this period must come from a further application of current plant breeding practices. We hope that advances in plant cell culture in the next decade will expand the relevance of this technology for plant breeders.

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