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Isolation of Agronomically Useful Mutants from Plant Cell Cultures

R. S. Chaleff

Summary. Enormous genetic variability is accumulated by plant cells proliferating in culture. Additional variability can be induced in cultured cell populations by exposure to mutagens. This pool of genetic diversity can be examined for agronomically desirable traits at two levels of differentiation. Populations of plants regenerated from callus cultures can be screened by conventional methods. Alternatively, selective culture conditions favoring growth of specific mutant types can be applied at the cellular level. The several characteristics that have been introduced by these methods to date are a harbinger of future contributions to be made by cell culture to the genetic improvement of crops.

It was not until the 1930's that several prior decades of research culminated in the successful propagation of plant organs and tissues in culture. Thereafter, progress in plant tissue culture was rapid. The techniques of culture in vitro were extended to many species and, aided by advances in the knowledge of plant hormones that were made in part through use of tissue culture, regeneration of plants from cultured tissues was achieved in the late 1950's. The first application of these developments was to the clonal multiplication of plants. The ability to regenerate large numbers of plants from masses of disorganized tissue (callus) proliferated in vitro and from cultured organs and axillary buds proved more efficient than conventional methods of asexual plant propagation. The lists in recent reviews (*1, 2*) of the hundreds of species that have been propagated through tissue culture document the extent to which this application of plant tissue culture technology has been developed and utilized.

In the 1960's, research in plant cell and tissue culture produced a number of achievements that individually represented significant technical advances and refinements. But when considered collectively these contributions effected a qualitative change in the conceptual view of the field. In 1960 Bergmann (*3*) demonstrated that single cultured cells plated in an agar medium would divide and form calluses. That same year Cocking (*4*) introduced an enzymatic procedure for isolating large numbers of protoplasts from higher plant tissues. In 1965 Vasil and Hildebrandt (*5*) demonstrated the totipotency of single plant cells by accomplishing the development of a complete and fertile plant from a single isolated somatic cell. Shortly thereafter Guha and Maheshwari (*6*) obtained haploid plants from immature pollen (microspores) contained within cultured *Datura* anthers. And in 1971 Nagata and Takebe (*7*) regenerated plants from cultured tobacco protoplasts. However, the turning point was in the realization that these discoveries, by making possible (albeit with only a small number of spe-

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cies) the experimental manipulations summarized in Fig. 1, conferred upon higher plants many of the attributes that had made microbes so amenable to genetic study. With the availability of large populations of physiologically and developmentally uniform haploid cells came the ability to select defined mutants. Genetic analyses could then be performed by conventional methods with regenerated diploid plants. These developments reached fruition in Carlson's (*8*) isolation of auxotrophic mutants from cultured tobacco cells.

Other opportunities for genetic experimentation with higher plants also became evident at this time. The technique of protoplast fusion is reviewed by Shepard and colleagues (*9*) and the possibility of introducing foreign DNA (genetic transformation) is considered by Barton and Brill (*10*) elsewhere in this issue. Accordingly, this discussion is confined to the application of plant cell culture to mutant isolation.

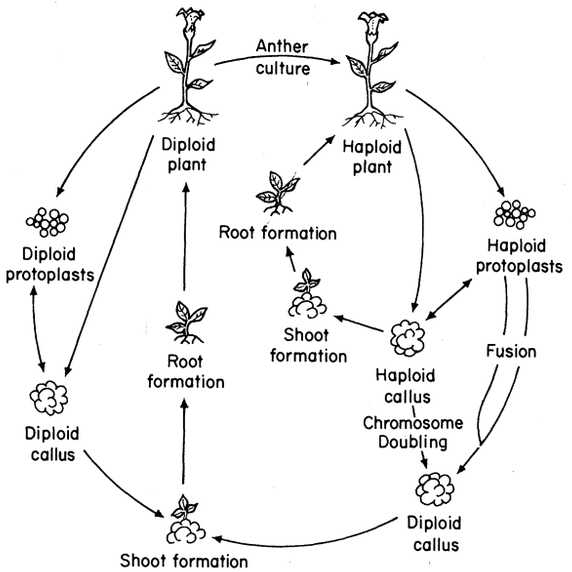

Fig. 1. Summary of experimental manipulations possible with *Nicotiana*, *Datura*, and *Petunia* (*13*). Haploid plants are obtained by culturing anthers or pollen of a diploid plant. Protoplasts capable of wall regeneration and subsequent cell division can be isolated from intact plant tissues and from callus cultures. The formation of shoots and roots from callus tissue is accomplished by altering the hormone composition of the medium.

Genetic Variability in Cell Cultures

The use of tissue culture for clonal propagation is based on the assumption that tissues remain genetically stable when excised from the parent plant and placed into culture. This assumption is largely valid when plant multiplication occurs by development of axillary buds or adventitious shoots directly from explanted organs. However, in cases in which shoot formation is induced from callus tissues, aberrant plants are often produced. Moreover, the frequency of such aberrant types increases with the length of time that the callus is maintained in vitro (*1, 11*).

The phenotypic variability observed among cultured cells and regenerated plants cannot be assumed to result only from genetic events, which include changes of nucleotide sequence and of chromosome number and structure. Physiological responses to the anomalous environment of the culture vessel and epigenetic changes also can contribute to such variability. Epigenetic events reflect altered levels of gene expression (resulting from abnormal operation rather than from mutation of regulatory mechanisms) that are relatively stable in that they persist through mitosis to be expressed by daughter cells. However, in contrast to altered phenotypes having a genetic basis, those resulting from epigenetic changes tend not to be expressed in regenerated plants or their progeny (*12, 13*). For the present, transmission through sexual crosses provides an acceptable criterion by which to distinguish genetic from epigenetic changes. However, this distinction provides us only with an operational definition that should not be applied too rigidly. Certain types of genetic change, such as gene amplification, can be unstable even through mitotic divisions in the absence of selection, and others, such as aneuploidy, often are not gametically transmitted.

Perhaps the earliest and most direct evidence of the genetic variability of cell cultures was furnished by nuclear cytology. Karyotypic variation provides visible proof of genetic heterogeneity within a population of cultured cells. Polyploidy, aneuploidy, and chromosomal rearrangements have been identified in cell cultures derived from a wide variety of plant species (*14*). But because the majority of these studies were conducted with cell cultures derived from tissue explants, they served to establish the fact, rather than the origin, of such variability. In such cases, one cannot determine whether unusual chromosome numbers or structures observed in cultured cell populations arose from the occurrence of mitotic irregularities in vitro or by multiplication of karyotypically abnormal cells present in the initial explant.

One means of resolving this question is by examination of cell cultures initiated from single cells. If variability is not generated during mitotic division in vitro, all members of the cloned population will be identical: any heterogeneity that is present in that population must have originated in culture. Consequently, the observation of cells of different chromosome numbers (diploid, polyploid, and aneuploid) in callus cultures obtained by cloning single cells of carrot (*15*) and tobacco (*16*) clearly demonstrated the occurrence of genetic variability in vitro. Pollen culture, and certain anther culture systems in which callus formation proceeds from the immature pollen contained within the cultured anther rather than from the surrounding somatic tissue, also provide single cells of a specified ploidy that can be stimulated to divide in vitro. Thus, the recovery of diploid and polyploid plants from pollen-derived callus cultures of *Oryza sativa* furnished additional evidence of abnormal mitoses in cultured cells (*17, 18*).

Another means of illustrating the occurrence of spontaneous genetic changes in cultured cells is by genetic characterization of novel cellular phenotypes. Much to their surprise, Chaleff and Keil (*19*) discovered that more than half of all tobacco cell lines isolated on the basis of resistance to the herbicide picloram were also resistant to hydroxyurea. Crosses with regenerated plants demonstrated that in the three cases analyzed, resistance to hydroxyurea was caused by a single dominant nuclear mutation. In two cases, the mutations conferring resistance to hydroxyurea (*HuR*) and to picloram (*PmR*) were genetically unlinked. Yet the callus culture from which the mutants were derived was sensitive to hydroxyurea and resistance arose only rarely among populations of sensitive cells and could be isolated only by deliberate selection. Moreover, the *HuR* mutations by themselves did not provide any detectable resistance to picloram, nor did they enhance the degree of picloram resistance conferred by the *PmR* mutations. Thus, the *HuR* mutations represented independent genetic events that occurred spontaneously in culture

and were recovered in the absence of any known selective pressure, although their appearance seemed related in some way to picloram resistance.

An exceptionally extensive and detailed analysis of the frequency and origin of variability in tobacco cell cultures was reported by Barbier and Dulieu (*20*). By constructing tobacco plants heterozygous at two loci for recessive mutations affecting chlorophyll synthesis, the occurrence of genetic events at either locus could be detected by the appearance of the recessive phenotypes in regenerated plants. The type of event responsible for the altered phenotype was then determined by crossing these plants with individuals homozygous for one or the other mutation. The frequency of genetic changes in populations of plants regenerated directly from explanted cotyledons via induced bud formation represented the amount of variability preexisting in cells of the intact plant (or arising during dedifferentiation and embryogenesis). The frequency of genetically altered plants in populations regenerated from callus cultures provided an estimate of the extent to which variability accumulated during propagation in vitro. Variability among plants regenerated from callus cultures was approximately ten times greater than among plants developed from cotyledonary buds. Interestingly, the greatest amount of variability was generated during the first passage in culture and little increase was observed in subsequent passages.

These several lines of evidence confirm that genetic variability arises spontaneously in plant cell cultures. But as yet we know nothing of the mechanisms by which these changes occur. They may simply be induced by a component of the culture medium. Alternatively, they may result from the breakdown of normal cellular or mitotic processes or from the activation of genetic systems, such as transposable elements, that are normally repressed. Another possibility is that such aberrant events occur at the same frequency in the intact plant, but that some of these mutations or genomic rearrangements either confer a growth advantage in culture that permits their selective proliferation or (as can be imagined for mutations affecting photosynthesis) selection against them is less stringent in vitro than in vivo.

On the one hand, the genetic instability of cultured plant cells can be considered a nuisance. It is more than likely that plants regenerated from cell cultures that have been maintained for a substantial period of time will carry deleterious genetic changes in addition to those of interest. Such excessive variation will confuse analysis of the desired trait and will necessitate outcrossing to incorporate that trait into an agronomically useful form. But on the other hand, the apparently mutagenic effects of cell culture provide a wealth of variability that can be screened for novel characteristics. By treatment with chemical or physical mutagens, the genetic variability of cell cultures can be even further enlarged. The ensuing discussion focuses on the difficulties and successes of attempts to date to isolate mutants of potential agronomic value from this newfound resource of genetic variability.

Screening Regenerated Plants for Desirable Characteristics

The variability present in cell cultures is ultimately visible in populations of regenerated plants. In some of the earliest studies of this type, differences were found in chromosome number, stature, auricle length, pubescence, and isozyme banding patterns among plants regenerated from cultured sugarcane cells (*21*). Because sugarcane plants are mixoploid (that is, not all somatic cells have the same number of chromosomes), some phenotypic variability was to be expected in addition to any variability that might be generated in vitro. Callus cultures established from mixoploid tissues will themselves be composed of cells of different chromosome complements from which plants of a range of chromosome numbers will be regenerated. It was not long before such plant populations were being examined for traits of agronomic significance. Screening for resistance to eyespot disease was performed by treating sugarcane plants regenerated from callus and suspension cultures with the toxin elaborated by *Helminthosporium sacchari*, the causative agent of the disease. An astonishing 15 to 20 percent of the regenerated plants proved resistant. Similarly, resistance to Fiji disease was expressed by 4 of 38 plants that had been regenerated from callus of a susceptible sugarcane variety. Regenerated plants possessing increased sucrose content and downy mildew resistance were also identified. Although no reports have appeared to date on the stability of these traits through sexual crosses, they have been maintained through several generations of vegetative propagation.

Enormous variability has also appeared among populations of potato plants regenerated from leaf mesophyll protoplasts. One study of 65 protoplast-derived clones (vegetatively propagated descendants of single plants) reported significant variation for 26 of the 35 morphological and physiological traits monitored (*22*). As in the case of sugarcane, some of this variability was manifested as resistance to diseases to which the parental cultivar was sensitive. Four clones resistant to early blight were identified by inoculating leaves of 500 regenerated plants with a crude toxin preparation obtained from cultures of *Alternaria solani*. Twenty clones of a population of 800 survived inoculation with the late blight fungus *Phytophthora infestans*. Resistance to both fungal diseases was expressed by subsequent vegetative generations (*23*).

Because the partial or complete infertility of most important sugarcane and potato cultivars makes sexual breeding of these species difficult, it is perhaps not surprising that the first reports of variability among plants regenerated from cultured cells came from studies with these species. Plants regenerated from callus cultures usually vary from the parental cultivar in only one or a few characteristics. This frequency of variation—sufficiently high that most individuals are altered in some way, but not so high that the majority of these individuals possess deleterious alterations—makes screening of regenerated plants a promising alternative to sexual breeding as a means of improving existing cultivars.

But the studies with vegetatively propagated crops leave unanswered two very important questions. First, although we know from the preceding section that genetic changes do occur in cultured cells, we do not know that the specific phenotypic alterations observed in the regenerated sugarcane and potato plants result from mutational events. And second, if these changes do represent genetic events, are they of a type—such as aneuploidy or chromosomal rearrangement—that by causing gametic inviability cannot be maintained in seed-propagated crops?

These questions have been addressed by several investigations on cereal species. A wide range of morphological abnormalities were observed among plants regenerated from oat callus. In many cases inheritance of these traits was followed through several generations of self-fertilization (*24*). Populations of plants regenerated from callus cultures initiated from rice seeds also displayed phenotypic variability. Differences from the parental variety were found in plant height, morphology, chlorophyll content, heading date, and fertility. Only 28

percent of the regenerated plants were not altered in at least one of these characters. The genetic basis of these phenotypic alterations was established by their expression in two subsequent sexual generations (*25*).

The genetic diversity of plants emerging from disorganized callus tissues provides the breeder with a means of introducing variability into established cultivars without the use of sexual crosses. But screening for desirable types still must be accomplished by conventional methods, which require large amounts of land and labor. In some cases another application of cell culture may provide a more efficient alternative.

Direct Selection in vitro

One of the major advantages afforded by cell culture for genetic experimentation with higher plants is that it makes possible direct selection for novel phenotypes from large physiologically and developmentally uniform populations of cells grown under defined conditions. Millions of cells, each representing a potential plant, can be cultured in a single petri dish 9 centimeters in diameter. Incorporation of toxic or growth inhibitory compounds in the medium allows growth only of the few resistant cells in the population, and from these isolates plants can ultimately be regenerated (Fig. 2). With recognition of the similarities between cultured plant cells and microorganisms came the expectation that all of the extraordinary feats of genetic experimentation accomplished with microbes would soon be realized with plants. But because of the many ways in which cultured plant cells are unlike microbes, these expectations thus far have not been well fulfilled.

Perhaps it is too often overlooked that, in contrast to microbes, which are autonomous units that have limited capacity for differentiation, plant cells evolved as components of highly complex and differentiated multicellular structures. Doubtless plant cells perform many of the same elemental activities and therefore have many features in common with unicellular organisms. However, as the multicellular plant (metaphyte) is the product of cellular functions and relationships that are unknown to the microbe, the cells of the metaphyte correspondingly must possess some properties and capabilities very different from those of the microbe. Some of these characteristics of cultured plant cells make difficult experimental manipulations that are taken for granted in microbial systems. For example, plant cells tend to grow in culture as aggregates. In addition, single plant cells cannot multiply when placed in an infinite volume of medium, but require a minimum inoculum density to achieve self-sustaining growth and division. And genetic analysis must await the time-consuming regeneration of plants and the completion of a very lengthy life cycle. But a unique feature of plant cell culture that imposes the most severe restriction on its use for genetic experimentation is that selection for a novel phenotype is conducted at a level of differentiation distinct from that at which phenotypic expression is ultimately desired. This last qualification has several consequences of special significance in selecting at the cellular level for genetic modifications of agronomic traits, which, for the most part, are products of differentiated cells, tissues, and organs present only in the whole plant.

The first constraint imposed by selection in vitro results from the fact that not all traits expressed by the whole plant are expressed by the cultured cell. Of course, one cannot select for modifications of a trait that is not expressed. This point is illustrated by the example of drought tolerance.

If a breeder identified a drought tolerant variety, he or she might look for deeper root penetration, altered control of stomatal closure, or a thicker cuticle as a basis for this phenotype. But these characteristics are functions not only of highly differentiated cells, but of the organization of such cells into complex organs and of interactions between these organs. At present, it is difficult to imagine how the expression of such traits could be elicited from single cells in culture. Accordingly, the somatic cell geneticist must accept that certain traits are exclusively whole plant functions and as such now lie beyond his or her reach. This is not to say that cell culture cannot be used to modify whole plant traits, such as drought tolerance, but only that this technique restricts one to approaches that involve selection for alterations of basic cellular functions. Thus, in applying cell culture to the problem of drought tolerance, one could not expect to select mutants with an altered root architecture. However, it might be possible to select cells capable of regulating their osmotic potential by production of osmotically active solutes. Protoplasts or cells possessing this capability could preferentially survive culture in a hypertonic medium.

Comparative studies on the susceptibility of whole plants and callus cultures to salt suggest that this trait can be effected by several different mechanisms—some acting at the cellular level and others only at the whole plant level. Callus cultures of the halophyte glasswort (*Salicornia*) are as sensitive to NaCl as are callus cultures of cabbage, sweet clover, and sorghum (*26*). But the relative degrees of salt tolerance of callus cultures of two barley species (*Hordeum vulgare* and *H. jubatum*) seem to correspond to those of the whole plants (*27*). These results indicate that one could select in culture for a mechanism of salt tolerance like that in barley, but

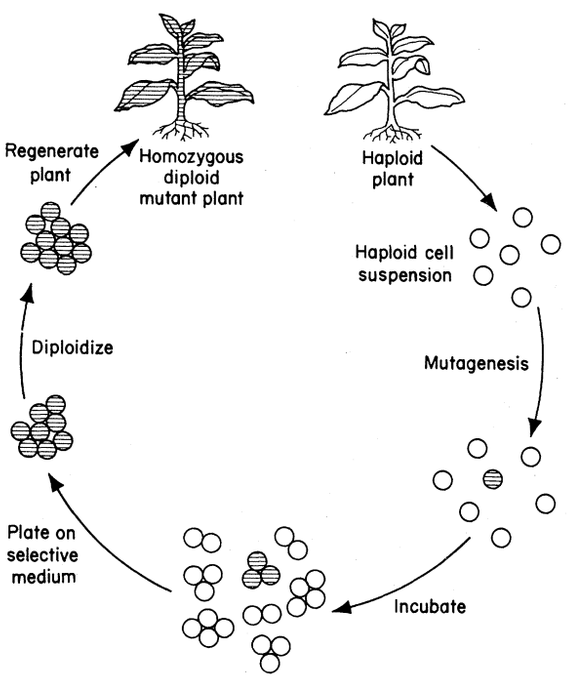

Fig. 2. Schematic representation of a general procedure for positive selection for mutants in plant cell cultures. Selection for recessive, as well as dominant, mutations is made possible by establishing cell cultures from haploid plants. Mutagenesis increases the genetic variability of the cell population, and incubation under nonselective conditions is necessary to allow expression of any newly induced mutant traits. After transfer to a medium that favors growth of mutant (filled circles) over nonmutant (open circles) cells, cultures composed largely or entirely of mutant cells are obtained. Finally, diploidization, occurring either spontaneously or in response to chemical treatment, is required for fertility of the regenerated plants.

not for one of the type operating in the halophyte. Salt tolerant cell lines have been selected from cell cultures of several species, but plants have been regenerated only in the case of tobacco. These plants and their progeny survived irrigation with a solution containing a salt concentration lethal to normal plants. However, inheritance of salt tolerance did not fit a conventional pattern and the possibility remains that tolerance is due to adaptive changes (such as an altered membrane composition) that are transmitted maternally in crosses rather than from a true mutation (*28*).

Other whole plant traits of agronomic importance such as yield, leaf canopy area, grain quality, and many types of pest resistance may prove less accessible by an in vitro approach. Not only are these traits not expressed by cultured cells, but our poor understanding of their molecular and cellular bases prevents identification of correlative cellular functions for which in vitro selection schemes might be devised. Rather ironically, cell culture grants us the general ability to select for mutant types, but precludes selection for many agronomically desirable features that are not expressed by cultured cells.

The second limitation of mutant selection in vitro can be stated as the converse of the first: Not all traits expressed by the cultured cell are expressed by the whole plant. The failure of a regenerated plant to express the novel phenotype of a selected cell line may have any of several causes. As mentioned earlier, phenotypic alterations resulting from epigenetic changes will usually be reversed by the processes of differentiation and meiosis. But expression of genetic alterations also can be developmentally dependent. Function of the mutated gene simply may be restricted to the state of differentiation represented by cultured cells. In some cases developmentally controlled repression of the mutated gene may be accompanied by the activation of distinct genes encoding enzymes with similar catalytic activities (isozymes). The developmental stage in which these nonmutant isozymes are synthesized will appear phenotypically normal, even though the plant harbors a mutant allele of the gene that is predominantly expressed in cultured cells. It is possibly as a consequence of the importance of polyploidization in their evolution that plants possess large numbers of isozymes (*29*).

To complicate matters further, plants regenerated from mutant cell cultures and in which the altered gene is fully expressed also can appear normal. Such is the case for tobacco mutants that were selected in vitro on the basis of resistance to isonicotinic acid hydrazide (INH), an inhibitor of glycine decarboxylation in the glycolate pathway of photorespiration. The function of the photorespiratory pathway in higher plants is not understood. But because it is a competitor of photosynthetic carbon fixation, elimination of this pathway is considered a possible means of increasing plant productivity.

As a first step toward devising genetic blocks that would decrease photorespiration, mutants resistant to INH were isolated from haploid tobacco cell cultures that had been irradiated with ultraviolet light. The growth of progeny seedlings from plants regenerated from INH-resistant cell lines was as sensitive to INH as was the growth of normal seedlings. However, callus cultures established from plants regenerated from these resistant cell lines and from their progeny were resistant. Thus, INH-resistance has a genetic basis and its expression is restricted to the cellular level (*30*). By direct biochemical assay glycine decarboxylase activities in both resistant callus cultures and in leaves of mutant plants were shown to be less sensitive to inhibition by INH than were the activities in the corresponding normal tissues. Cosegregation of INH resistance and an altered glycine decarboxylase activity in sexual crosses strongly suggest that the reduced sensitivity of this enzyme complex to INH is the basis for the resistance phenotype (*31*). But although this biochemical alteration appears in both callus and plant, only callus and not seedling growth displays resistance to INH.

Disease resistance was the first trait of agronomic interest for which selection at the cell level was rewarded by expression by the whole plant. In selecting for disease resistance in vitro, cells are plated on a medium supplemented with a lethal concentration of the disease toxin. Consequently, this procedure is applicable only in cases in which a toxin produced by the microbial pathogen is primarily responsible for the disease symptoms. The wildfire disease of tobacco seemed to provide just such an experimental system. This disease is caused by a bacterial pathogen, *Pseudomonas tabaci*, which elaborates a toxin that produces chlorosis of leaf tissue. Resistant cell lines were selected by exposing populations of mutagenically treated haploid tobacco protoplasts or cells to a growth-inhibitory concentration of methionine sulfoximine, an analog of the wildfire toxin that elicits the same characteristic disease symptoms as does the natural bacterial toxin (*32*). The chlorosis that normally develops on leaves of the parent plant in response to inoculation with either *P. tabaci* or a solution of methionine sulfoximine did not appear following inoculation of leaves of plants regenerated from three resistant cell lines. Moreover, resistance segregated among progeny of sexual crosses in accordance with conventional Mendelian patterns. But the success of this experiment was only partial. Although inoculation with either methionine sulfoximine or *P. tabaci* did not cause bleaching of mutant leaf tissue, small necrotic spots did develop on leaves of mutant plants at the point of inoculation with the bacterial culture. These lesions resembled those obtained from infection of tobacco with *P. angulata*, a variety of *P. tabaci* that does not produce toxin. Therefore, it is apparent that selection for resistance to methionine sulfoximine yielded plants that were insensitive to the action of the toxin itself but that were still susceptible to other deleterious effects of bacterial infection.

Selection for toxin resistance among cultured cells has also been used to isolate plants resistant to southern corn leaf blight (*33*). *Dreschslera maydis*, the causal agent of the disease, produces a toxin to which both maize plants carrying the Texas male sterile cytoplasm (*cms*-T) and callus cultures derived from such plants are susceptible, but to which plants and callus possessing normal nonsterile cytoplasm are resistant. Because male sterility is of great advantage for hybrid seed production, it is desirable to introduce resistance to the fungal pathogen into the male sterile cytoplasm. To this end *cms*-T callus cultures were transferred to medium supplemented with a crude toxin preparation. Toxin-resistant cell lines were isolated and plants regenerated. All plants regenerated from callus cultures that had been maintained on selective medium for five passages or more were resistant to the toxin. But the majority of these toxin-resistant plants were male-fertile and the sterility of the remainder was due to something other than *cms*-T cytoplasm, since none of these plants could function as either a male or female parent, and in most cases floral organs were deformed. It is probable that the sterility of these plants resulted from random aberrations generated during propagation in vitro. Both the fertility and toxin response of progeny produced by crosses with toxin-resistant regenerated plants always resembled that of the maternal parent. These progeny were also infected with *D. maydis* spores, and in all cases the

reactions to the fungus and to the partially purified toxin solution were the same. Thus, in contrast to the results obtained by selection of methionine sulfoximine resistance in tobacco cell cultures, selection for resistance of maize callus to the *D. maydis* toxin produced plants that were also resistant to the causal organism. These experiments were repeated by others with, for the most part, similar results (*34*). However, a curious difference in the latter experiments was the recovery of fertile toxin-resistant plants from control *cms*-T callus cultures that had never been subjected to selection. Nevertheless, in neither set of experiments was the desired product of a male-sterile disease-resistant maize plant obtained.

Perhaps in the immediate future greater practical benefit is to be realized from the application of in vitro selection methods to the isolation of plant mutants altered in the control of amino acid biosynthesis. The nutritional quality of a food crop may be substantially improved by genetic modifications effecting increased production of amino acids that are at present limiting to protein quality.

A means of selecting such mutants is provided by the feedback sensitivities of amino acid biosynthetic enzymes. In maize, the activities of aspartokinase and homoserine dehydrogenase, two enzymes of the pathway for the synthesis of the essential amino acids lysine, methionine, threonine, and isoleucine, are inhibited by lysine and threonine, respectively. Therefore, in the presence of excess lysine and threonine, methionine biosynthesis is interrupted and cell growth is inhibited. However, mutant cells producing an altered enzyme that is insensitive to end-product inhibition will be able to grow under these conditions.

A cell line resistant to growth inhibition by a mixture of lysine and threonine was isolated from maize callus cultures that had been treated with the mutagen sodium azide (*35*). Fertile plants were regenerated and genetic crosses demonstrated that resistance was inherited as a single semidominant nuclear mutation. Homozygous mutant kernels contained as much as 100-fold more free threonine than did normal kernels. The levels of free methionine, serine, and proline were elevated three- to fourfold and the free pool sizes of the remaining amino acids were essentially unchanged. Although the threonine content of seed protein was not altered, the magnitude of the increase in free threonine resulted in a 50 percent increase in the total amount of threonine present in mutant kernels.

Selection among cultured cells has also been successfully used to enhance the tolerance of a plant for a particular herbicide. The effectiveness of herbicides is based on their ability to discriminate between weed and crop species. Although traditionally it has been left to the chemist to synthesize compounds that display this specificity, differential responses to a herbicide can also be achieved by introducing tolerance into the crop species by genetic means. This genetic approach should broaden the spectrum of applicability of existing herbicides and thereby spare the enormous expense of developing and licensing new herbicides.

Several tobacco mutants resistant to picloram were isolated by plating cultured cells on herbicide-supplemented medium (*36*). Plants regenerated from five isolates were analyzed genetically. In three cases (*PmR1*, *PmR2*, and *PmR7*) resistance resulted from single dominant nuclear mutations and in two cases (*PmR6* and *PmR85*) from single semidominant nuclear mutations. Additional crosses established genetic linkage between *PmR1* and *PmR7* and assigned *PmR6* and *PmR85* to distinct linkage groups (*13*). Growth of callus initiated from plants homozygous for the *PmR1* mutation was 100-fold more tolerant of picloram than was growth of normal callus. The expression of increased tolerance by mutant plants is illustrated in Fig. 3.

Conclusion

The genetic variability generated during proliferation of plant cells in culture and that induced by mutagenic treatment can be examined for desirable traits at two levels of differentiation. Regenerated plants can be screened by conventional methods. But even in cases in which visual screens are employed, this procedure is both labor- and land-intensive. Alternatively, novel phenotypes can be selected directly at the cellular level by defining culture conditions that favor growth of the variant and discriminate against growth of normal cells. This is a potentially powerful method that permits enormous numbers of genomes to be scrutinized both rapidly and rigorously within the dimensions of a culture vessel. Unfortunately, however, certain features of selection in vitro preclude its

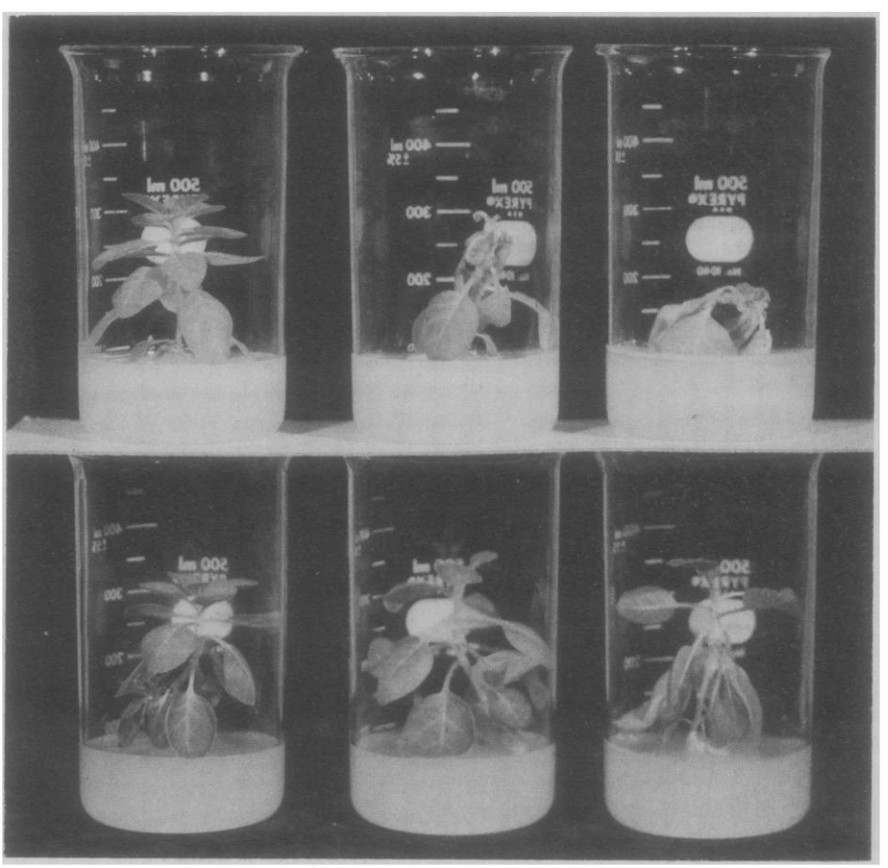

Fig. 3. Effects of picloram on normal (top) and homozygous mutant (*PmR1*/*PmR1*) (bottom) tobacco plantlets. The plantlets were grown axenically for 8 weeks. Picloram was then added to several of the beakers to the following final concentrations: no picloram (left), 1 μ*M* (center), and 5 μ*M* (right) (*13*).

application in many cases and limit one to the more laborious procedure of screening regenerated plants.

One limitation of mutant selection in vitro is that it can only identify modifications of traits that are expressed at the cellular level. The developmental complexity of higher plants makes this restriction rather severe. Many traits of agronomic importance are the products of the organization of highly differentiated cells and, therefore, do not appear in culture. And, because of our rather primitive understanding of their molecular and cellular bases, correlative functions of these traits that may be expressed at the cellular level have not yet been defined. Thus, although certain agronomic traits, such as tolerances for heavy metals, salt, herbicides, and extremes of soil *p*H, may prove accessible by an in vitro approach, others, such as yield, lodging resistance, and times to flowering and maturity, are, at least for the moment, beyond the reach of the somatic cell geneticist.

Another sine qua non of mutant selection in vitro that greatly limits its application is the ability to regenerate plants from cultured cells. In contrast to the production of regenerated plants for screening, identification of mutant types at the cellular level actually requires several successive passages on selective medium. Therefore, the capacity to regenerate plants must be retained by cells throughout prolonged periods in culture. It is primarily for this reason that most successful selections of mutants from cell cultures have been accomplished with tobacco. Sustained morphogenetic capacity still tends to be the exception rather than the rule among the major crop species. Even in the case of cereals, where significant advances have recently been made (*37*), morphogenetically competent cell cultures are difficult to obtain and do not grow as dispersed and homogeneous cell populations, but as highly organized aggregates, which are far from ideal for mutant selection.

Notwithstanding the present stage of development of the art of tissue culture, direct selection for mutants from cultured cells is a valuable technique for crop improvement. However, its suitability must be evaluated independently for each application. In some cases, screening of regenerated plants will provide a more efficacious means of identifying desirable phenotypes in the pool of variability produced in cell culture. But as the techniques of cell culture are refined and extended to more species, a corresponding increase can be expected in the contributions of in vitro selection to the genetic improvement of crop plants.

References and Notes

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