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CELLULAR APPROACHES
TO
AGRICULTURAL GENETICS

Progress Report

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ABSTRACT

The intent of this project is to define conditions for the in vitro culture and subsequent regeneration into whole plants of somatic cells of important crop species. Work during this year has focused upon defining the role of several variables in regeneration of cultures derived primarily from potatoes, barley and long term tobacco cultures. Specific variables which were examined included: source of the initial explant for in vitro culture, effects of various hormones (primarily 2,4-D and ABA), effects of alterations of the basic mineral salts medium, effects of various physical shocks and effects of selection and subculture of callus approximating a desired morphological type.

Further research is focused on defining the response of plant cells to a range of mutagenic, carcinogenic and teratogenic agents. Initial work in this area has developed and characterized a haploid cell line of tobacco capable of responding quantitatively to these agents.

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The approach of this work is almost entirely empirical. Regeneration of entire plants from established cell cultures occurs only in a few species of higher plants, and even in those cases we don't know why it happens. We have no generalizable methods or procedures. It is the intent of this work to develop reproducible techniques for plant regeneration which can be applied routinely to a range of cultivars of the important crop species. Thoroughness is essential in this work because the literature contains a number of spurious reports of regeneration which cannot be reproduced. Our aim is to make regeneration a routine technique and not a fortuitous event. Considerable attention has been given to experimental detail.

The process of regeneration in those species where it can now be accomplished (e.g., tobacco and carrot) occurs in response to a single alteration in the medium (e.g., removal of auxin or addition of a cytokinin). These cases appear to occur as single step phenomena. The work done under this contract has demonstrated that regeneration in many species is a complex problem of developmental biology. Regeneration is a process actually composed of a number of discrete sequential steps. We have come to recognize a few of these landmarks along the process, and have determined that different steps in the process require different stimuli for completion. Hence, coordinant with our efforts to vary media composition outlined

below, we have attempted to use these landmarks in the process of achieving regeneration.

The following items summarize the significant progress achieved under this contract during the past year:

1. Regeneration of barley plants from suspension cultures.

Successful regeneration of viable plants from single cells or small clumps of cells has not previously been reported with cereal grain species. We have successfully accomplished, within the past year, the regeneration of entire barley plants from suspension cultures of this species, and we are defining methods for routinely regenerating barley and other cereal grains. The two important components which permitted us to accomplish this regeneration were: (a) the use of tissues derived from immature ovaries, and (2) the use of certain interspecific hybrids between Hordeum vulgare and Ht. jubatum, a wild perennial barley species. The importance of these two components (i.e., ovarian tissue and hybrids with normally perennial wild species) is being further examined. Two other important observations have emerged from this work; the occurrence of different cellular factions and the presence of a transmissible factor which inhibits mitotic activity. The generation of a liquid suspension culture from primary callus tissue of barley was initially observed to follow a

reproducible pattern. After introduction into a liquid medium, two distinct cellular populations become evident. One fraction is multicellular and meristematic in nature, while the second is not meristematic and is composed of autonomous cells and clumps of cells. Initial cytological observations of the autonomous fraction revealed a total absence of mitotic activity. As the suspension cultures aged this autonomous cell population produced numerous aberrations including multi-nucleate cells, giant cells and some presumed anucleate cells. When rapidly growing cells of tobacco are cultured on medium previously utilized for the culture of barley cells, the growth of the tobacco is markedly depressed. We feel that a factor(s) which inhibits cell growth or division may be produced on these barley cultures and that this factor(s) may be the cause of earlier negative results with this species.

2. Recurrent selection can be utilized to successfully regenerate difficult materials.

It has been suggested that the gradual decline in organogenic potential observed in some cell lines results from an accumulation of genetic defects that block morphogenesis. Cytological studies reveal that the ploidy of cultured cells is usually unstable and that the number of polyploid and aneuploid genotypes tends to increase with duration in culture. But it has also been shown callus

cultures can produce organs with both polyploid and aneuploid meristems. In other instances the declining embryogenic capacity of a long-term carrot cell line was restored by treatment, in one case with a cold shock and in another by treatment with very low levels of kinetin. These observations argue against the contention that the declining morphogenetic capacity of older cultures is due to genetic alterations that block totipotency. An alternative model might be that rather than an irreversible loss of totipotency, cultured cells lose the competence to respond to conditions normally used to induce organogenesis. Competence itself could be a differentiated function associated with a particular physiological or developmental state. As such, the loss of competence would simply reflect the continued dedifferentiation of cultured cells. It becomes very important in terms of regeneration that we learn to recognize and manipulate the different developmental states of cells in cultures.

For an experimental system we have chosen a tobacco cell line that has been cultured for 15 years. This is the cell line used by Filner and by Widholm for physiological and genetic studies. It lost the ability to regenerate plants more than six years ago. (Range of chromosome number observed is approximately 45-100 mode is in high 70's (77-79) but 5-10% still have a "near-diploid" chromosome number probably within counting error.) If the loss of totipotency

results from developmental rather than genetic alterations in cultured cells then this cell line should be an appropriate model. (We have also worked with cell lines of potato that were grown in suspension for three months and have been in culture for more than two years.)

We know very little about the developmental changes that take place in cells during the process of establishing and maintaining cell cultures. To some extent this transition from a differentiated tissue is achieved by supplying the cells with required nutrients and growth factors. And to some extent it involves a selection process for cells that are capable of rapid unorganized growth under the culture conditions employed. In other words, even though we don't know anything about the intermediate stages in this developmental transition we can achieve the desired growth form by selecting for its characteristics. We thought if the loss of totipotency was not due to genetic blocks then we might be able to reverse such a developmental sequence by using the same kind of selection process. Instead of incubating callus under inducing conditions and hoping to make the transition in one step on a single incubation period, we might select for intermediate stages that would in turn be more responsive to the regeneration medium. We incubated callus from Filner's tobacco cell line on the medium normally used to induce regeneration with tobacco. After six weeks the

callus had a very heterogeneous appearance. Most of the callus remained friable and unorganized but some sectors were very pale green and more compact. These sectors were selected and cultured again on the same inducing medium. After incubating for four to six weeks we again selected the greenest and most organized portions of each callus. On the third round of this type of selection some calluses formed small abnormal leaf-like structures. With each round of selection the teratoma-like structures produced have become more normal in appearance. First we find abnormal leaves - single and in groups, but no stem development and no evidence of apical growth. Later, with continued selection for 5-7 generations, we begin to find shoot-like organization. This progress is very encouraging since with successive rounds of selection we are recovering variants that are capable of increasing levels of organization. We do not know whether the variants being selected are genetic or epigenetic in nature. None of these presumptive shoots have continued to develop when transferred to media usually used to induce roots. Thus, no normal plants have been recovered yet. These are still not very normal in appearance for they have small internode distances and the leaves are small and abnormally shaped. However we have recently on at least five occasions observed root formation. This proves the genetic information is still there for root formation. Perhaps

coincidentally, perhaps not, it has taken 6-8 months, 4-6 generations of selection under rooting conditions to begin to see root formation. Thus far roots appear to arise from the callus and not from shoots.

A similar approach has been utilized with the potato cultures, yielding small plantlets following several rounds of selection. Progress with this procedure is slow but steady with increasingly organized sectors arising in successive transfer generation.

3. Initial culture or maintenance of tissues with NAA or 2-4,D inhibits the potential of subsequent regeneration.

New callus cultures from several different tissues of potato were initiated and maintained on seven different types of media both with and without 2-4,D. These cultures were each subsequently tested for regeneration on three different regeneration medium. These cultures which had not been initiated or maintained in the presence of either 2-4,D or NAA produced uniformly greater numbers of shoots on regeneration medium. Similar results were obtained with cultures of tomato.

4. The growth characteristics and responses of a haploid tobacco cell line have been characterized.

We have developed a cell line of haploid tobacco in order to quantitate

the responses of plant cells to mutagenic, carcinogenic and teratogenic agents. The cell line displays a sigmoidal growth pattern, can be initiated with a low inoculum and has a 36 hour cell cycle. Patterns of cell growth have been characterized by volume, weight, total protein, total DNA and by O. D. The number of viable cells as determined by several criterion is $90 \pm 1\%$. Curves of killing concentrations of several agents are now being characterized as well as the production of defined genetic variants.